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Since the coliform group of organisms is considered to be less resistant to chlorine than some bacterial and viral pathogens, the utility of both yeast and acid-fast organisms as potential indicators of disinfection efficiency was evaluated. In most laboratory studies these two groups of organisms were represented by Candida parapsilosis and Mycobacterium fortuitum, respectively. The relative resistance of the test organisms to free chlorine was: acid-fast> yeast>coliforms. The increased chlorine resistance of these organisms appeared to be the result of the thickness and impermeability of the cell wall. It was concluded that the primary mode of action of chlorine in disinfection was disruption of the cell membrane with a resultant change in cell permeability and physical damage to the cell DNA.

Resistance to ozonation was also studied. Variations in pH between 5 and 10 did not significantly affect organism survival of either yeasts or acid-fast organisms using constant ozone residuals, while increasing temperatures increased the inactivation of both organisms. Large volume sampling and enumeration techniques were developed for the yeasts and acid-fast organisms using membrane filtration which enabled the enumeration of these organisms at the relatively low densities found in finished drinking water.

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

This paper describes enumeration techniques for alternative bacterial indicators of pollution and evaluates their resistance to chlorine and ozone disinfection. This provides a much more meaningful measure of the effectiveness of disinfection of wastewater and water supplies, thereby limiting the introduction of contaminants into our waters and helping to protect the quality of our water supplies.

Francis T. Mayo
Director
Municipal Environmental Research Laboratory

ABSTRACT

Since the coliform group of organisms is considered to be less resistant to chlorine than many pathogens, including viruses, the utility of both yeast and acid-fast organisms as potential indicators of disinfection efficiency was evaluated. In most laboratory studies, these two groups of organisms were represented by *Candida parapsilosis* and *Mycobacterium fortuitum*, respectively.

Yeast and acid-fast organisms were found to be consistently present in raw municipal wastewater at a density of approximately 10^4/100 mℓ; fecal and total coliforms were present at a density of about 10^6/100 mℓ and 10^7/100 mℓ, respectively. Wastewater chlorination, following secondary treatment, reduced the density of these organisms by 1-3.5 logs. Laboratory studies involving lime/alum coagulation were observed to remove yeasts and acid-fast organisms by 90-99 percent. Removal of acid-fast organisms by sand filtration was from 40-70 percent, and yeasts and coliforms were removed by at least 90 percent.

The relative resistance of the test organisms to free chlorine under continuous flow conditions was: acid-fast > yeast > coliforms, using both mixed pure-cultures and diluted clarified activated sludge effluent. *M. fortuitum* occasionally survived breakpoint chlorination conditions. Relative resistance to ozonation was: *M. fortuitum* > poliovirus > *C. parapsilosis* > *Escherichia coli* > *Salmonella typhimurium*. Variations in pH between 5 and 10 did not significantly affect percent organism survival of either yeasts or acid-fast organisms under constant ozone residual while increasing the temperature from 9° to 40°C increased the inactivation of both organisms.

Large volume sampling techniques for the enumeration of yeasts and acid-fast organisms were developed for membrane filtration of 1 ℓ water samples. Yeasts and acid-fast organisms were enumerated in finished drinking water at densities of 1.5 and 2.2/ℓ, respectively. Increased resistance of *M. fortuitum* to free available chlorine was judged to be the result of the impermeability of its cell wall; the increased resistance of *C. parapsilosis* appeared to be the result of the thickness and rigidity of its cell wall. It was concluded that the primary mode of action of chlorine in disinfection was disruption of the cell membrane and with a resultant change in cell permeability, and physical damage to cellular DNA.

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SECTION 1

INTRODUCTION

OVERALL OBJECTIVES OF THE STUDY

The practice of disinfection as applied to water treatment and to wastewater effluents has generated considerable interest in recent years. At the present time, the major disinfectant in use in the United States is chlorine. The major objective of chlorination, or of disinfection in general, is to produce a finished product that is acceptable from a public health standpoint.

In order to assess the efficiency of disinfection in terms of destruction of pathogens, the usual approach is to utilize a bioindicator believed to be at least as resistant to disinfection as the most resistant pathogens. To be a satisfactory bioindicator, an organism or group of organisms must meet certain requirements (1,2). For example, a bioindicator should be rapidly and unambiguously quantifiable in water and wastewater samples by simple and easily applied techniques. The utility of the bioindicator would be enhanced if it also could serve as an indicator of fecal contamination.

Since it appears that total and fecal coliforms, currently the most commonly used groups of bioindicators, do not meet the criterion of being as resistant to chlorination as the most resistant pathogens, the suitability of coliforms for evaluating chlorine disinfection efficiency may be seriously questioned. This is particularly true where wastewater reuse schemes are being considered; in such situations protection of the public health is paramount.

The coliform organisms have been useful in the past in providing information on the potential presence of bacterial pathogens in waters and wastewaters. However, in light of their inability to satisfy the intended purpose in certain situations (3), it would seem appropriate to reevaluate the application of coliform organisms as bioindicators on a case-by-case basis. In this approach, the most appropriate indicator organism could be selected on the basis of its purpose and the information required, e.g., the inactivation of resistant viral pathogens by chlorination. It was with this purpose in mind that this study was undertaken.

Representatives of two groups of organisms, yeasts and acid-fast bacteria, were isolated from chlorinated wastewater secondary effluent. Early studies indicated that these organisms were substantially more resistant to chlorine and somewhat more resistant to ozone than were coliforms and, as a result, could be considered as possible new indicators of disinfection efficiency.

It was the purpose of this research to critically analyze these groups of organisms as to their utility as indicator organisms.

SUMMARY OF PREVIOUS WORK

In a previous report to the Office of Research and Development of the U.S. Environmental Protection Agency, dated February 1974, a detailed literature review concerning the applicability of the currently used fecal coliform index for determining disinfection efficiency was presented (4). The basic conclusions drawn from the literature review and other studies from this initial phase of the project are briefly restated here.

The literature indicates that the currently used coliform group of organisms is too sensitive to chlorine to be a totally reliable indicator of the potential presence of chlorine resistant pathogenic agents. For example, existing data suggest that many enteric viruses are more resistant to chlorine than coliforms. While the resistance of vegetative bacteria to chlorine is varied, the majority exhibit similar or less resistance to chlorine than coliforms; spore-forming bacteria, however, are found to be considerably more resistant to chlorine than vegetative cells. The resistance of cyst-forming pathogenic protozoans to chlorine has also been reported to be greater than the resistance of coliforms in laboratory studies; however, since a strong dependency of cysticidal chlorine dose on cyst density was noted, comparisons with field conditions may be inappropriate.

In cases where the presence of waterborne acid-fast bacilli are of concern from a public health standpoint, such as with *Mycobacterium tuberculosis*, the nature of the cell wall appears to confer a greater degree of chlorine resistance than that associated with the enteric bacteria. A review of the literature covering the relative resistance of certain acid-fast and other organisms is presented in Appendix A. The basic conclusion of this literature review was that coliforms are not entirely acceptable indicators of disinfection efficiency, based on the possible presence of pathogenic organisms which are more chlorine-resistant than the coliform group.

Studies to identify a new indicator of disinfection efficiency were initiated by the examination of organisms isolated from chlorinated wastewater and certain pure cultures obtained from the Department of Microbiology at the University of Illinois at Urbana-Champaign (5). Isolates included both gram-positive and gram-negative rods, cocci, and branching forms with white, orange, and yellow pigmentation. Gram-positive spore-forming rods were eliminated from consideration as potential indicator organisms due to the uncertainty of sporulation as affected by environmental conditions. The use of a wide variety of enrichment media and incubation conditions, as well as the continuous culturing and isolation of organisms over a period of many months, facilitated the establishment of a variety of organisms present in chlorinated water and wastewater. The isolates were screened for their resistance to free available chlorine using chlorine demand free water (CDFW) at pH 7. The criterion for classification as a chlorine resistant isolate was survival after 30 min exposure to 1 mg/ℓ free chlorine. According to this standard, the isolates displaying the highest degree of chlorine resistance were yeasts and acid-fast bacilli. Of these isolates, one yeast, previously reported as No. 30, and two acid-fast bacilli, previously reported as Nos. 132 and 134, were selected for further study; these organisms were identified subsequently as *Candida parapsilosis*, *Mycobacterium phlei*, and *Mycobacterium fortuitum*, respectively.

Pure culture inactivation experiments were performed with these organisms using free chlorine at residuals of 0.1-2.0 mg/ℓ in pH 7 chlorine demand free phosphate buffer (CDFB) at 20°C. A comparison of these data with those published in the literature for several enteric viruses led to the conclusion that yeasts and acid-fast organisms show sufficient resistance to chlorine to warrant further study.

Substantial effort was directed towards the development of enumeration techniques for acid-fast organisms and yeasts. After examination of several media and selective agents, it was determined that acidified yeast extract-malt extract-dextrose agar plus several selective and enrichment agents were adequate for the enumeration of yeast organisms (5,6). The development of an acid-fast enumeration technique was approached in three ways, i.e., the evaluation of a pre-treatment step, selective media, and a final staining procedure. The results of these three approaches when combined produced a satisfactory method for the enumeration of acid-fast organisms (5,6). More recent modifications are discussed in Section 4, Materials and Methods.

The results of the second phase of the overall study were covered in a report to the U.S. Army Medical Research and Development Command and to the U.S. Environmental Protection Agency, prepared in July 1975 and published in 1977 (5). The basic conclusions from this report are summarized below.

To ensure the consistent presence of the acid-fast and yeast organisms in wastewater, a detailed study of acid-fast organisms, yeasts, and fecal coliforms in raw domestic wastewater and treated effluents was performed. In addition, a study of the occurrence of acid-fast and yeast organisms in fecal matter was performed so as to identify their source when these organisms are isolated from wastewater. From these studies, it was found that yeasts and acid-fast organisms are consistently present in raw and treated domestic wastewater. However, while fecal matter was a fairly consistent and reliable source of yeasts, it could not account for the high densities of acid-fast organisms observed in raw wastewater. To further define the source of these organisms in wastewater, as well as in surface water, several of the more common acid-fast and yeast organisms isolated from wastewater were identified as to genus and species.

To avoid the dangers associated with comparing the response of indicator organisms and pathogenic organisms from separate chlorine inactivation experiments performed under possibly differing experimental conditions, mixed culture experiments were undertaken using two different yeasts, four different acid-fast organisms, *Escherichia coli*, *Salmonella typhimurium*, and poliovirus type 1 (Mahoney). In these studies, varying conditions of pH and temperature were evaluated in an attempt to further identify the relative resistance of these organisms to inactivation by free chlorine. These experiments confirmed the superior chlorine resistance of the proposed indicator organisms.

Studies with inorganic chloramine were performed using pure cultures of one yeast, two acid-fast organisms, and *E. coli* in CDFB at pH 7. These experiments indicated that the resistance of the yeast and the acid-fast organisms to chloramine was superior to that of *E. coli*. An investigation to test the proposed indicator organisms under conditions of ozone disinfection was initiated, also.

BACKGROUND AND OBJECTIVES OF THE REPORT

This study was initiated 1 December 1969 under a contract (17060 EYZ) from the U.S. Environmental Protection Agency. A report covering the period 1 December 1969 through 30 April 1972 was submitted in September 1972 and subsequently published by the U.S. Environmental Protection Agency in February 1974 (4). During the period of 1 May 1972 through 31 December 1977, the study was funded equally by the U.S. Environmental Protection Agency and the U.S. Army Medical Research and Development Command under interagency agreement EPA-IAG-D6-0432. A report covering the period 1 May 1972 through 31 April 1975 was submitted to the funding agencies in July 1975, and was published as a report of the U.S. Environmental Protection Agency in August 1977 (5). The present report covers the period 1 May 1975 through 31 December 1977.

Work covered in the previous reports (4,5) finalized the first two phases of the study which involved a search for a valid indicator organism of disinfection efficiency, development of enumeration methodology, verification of increased resistance of the organism to disinfection, confirmation of the presence of the organism in wastewater influents and effluents, and other laboratory studies exploring the utility of the proposed new organisms. This has been reviewed briefly under "Summary of Previous Work" in this report.

The final phase of the study, as discussed in this report, includes additional work on the development of enumeration techniques for use in treated water samples of larger volumes. Using methods as developed, a detailed study of acid-fast bacteria, yeasts, and fecal and total coliforms was undertaken in a wastewater treatment plant, the associated receiving stream, and a downstream water treatment plant using the same stream as a raw water source. From these studies, over a period of 9 months, earlier results were confirmed noting the consistent presence of the proposed indicator organisms in water and wastewater samples. Analysis of the variation between the organisms confirmed the increased resistance of the acid-fast and yeast group of organisms to wastewater treatment and disinfection when compared with the coliform group.

To further determine the occurrence of the proposed indicator organisms, a separate study, using large-volume samples, was performed in a water treatment plant and its associated distribution system. The results of this investigation confirmed the indicator organism resistance relative to coliforms and, indirectly, showed that a well operated water treatment plant is capable of achieving a moderate degree of removal of acid-fast and yeast organisms.

Since the densities of the proposed indicator organisms observed in field studies were generally low, it seemed desirable to confirm that removal of the organisms in water treatment unit operations, prior to disinfection, was not so great as to produce negligible indicator populations at the influent to the disinfection process. Laboratory coagulation-flocculation studies using lime, alum, or ferric chloride as coagulants, and sand filtration experiments were performed.

To verify the resistance of the proposed new indicator organisms to free and combined chlorine, continuous flow chlorination studies were performed using mixed pure-cultures in CDFB; diluted secondary wastewater effluent was also used. Results confirmed the data from batch studies in which increased resistance of the proposed indicator organisms to free and combined chlorine was observed, as compared to *E. coli*.

Because ozone is frequently used as a disinfectant for water and wastewater in European countries, and may be a substitute for chlorine in the U.S. under certain conditions, studies were performed to determine the ozone sensitivity of the proposed indicator organisms, *E. coli*, poliovirus type 1 (Mahoney), and *S. typhimurium*. While the acid-fast organism exhibited maximum resistance to ozone, the yeast organism appeared to be less resistant than poliovirus.

Experiments to determine the mechanism of inactivation of *E. coli* and the proposed new indicator organisms by free chlorine were also undertaken.

SECTION 2

CONCLUSIONS

Additional information has been presented regarding the potential use of acid-fast organisms and/or yeasts as indicators of chlorination efficiency. In a study of a wastewater treatment plant (WWTP) at St. Joseph, Illinois, its associated receiving stream, and a downstream water treatment plant (WTP) at Oakwood, Illinois, the proposed indicator organisms were enumerated. Compared with the coliform group, the yeast and acid-fast organisms were found to be more resistant to secondary wastewater treatment and chlorination. A 24-hr study to determine the diurnal variation based upon organism density in the WWTP revealed that yeast and acid-fast organisms were consistently present at a density of approximately 10^4/100 mℓ in raw wastewater. Fecal and total coliform densities in the raw wastewater were consistently higher than the proposed indicators by an order of 2-2.5 and 3 logs, respectively. Reduction of organism density as a result of chlorinating the secondary wastewater effluent was observed to be in the range of 1-3.5 logs. The relative resistance of the four organism groups to chlorination was found to be: acid-fast > yeast > total coliforms > fecal coliforms.

The occurrence of these four groups in the receiving stream for the WWTP effluent was also monitored. It was found that the wastewater effluent did not significantly change the quality of the water in the receiving stream. Physical-chemical parameters monitored above and below the effluent outfall supported this conclusion. Actual organism densities in the stream exhibited seasonal variations, being generally lower during colder periods than during warmer periods.

Recovery of all organisms from a holding reservoir, serving as the raw water supply of the WTP, was much lower than their respective densities in the river system supplying the reservoir. The majority of organisms in the raw water were removed by coagulation/flocculation/prechlorination within the WTP. Further reductions in organism density were observed after sand filtration. Yeasts were not recovered from any finished water samples in this WTP; acid-fast organisms were recovered from only one out of seven finished water samples.

One-liter volume sampling for enumeration of the proposed indicator organisms, yeasts and acid-fast bacteria, was found to be feasible. Techniques were developed to allow membrane-filtration of 1 ℓ samples of both raw and treated drinking water at a water treatment plant. Utilizing these techniques, the water treatment plant at Decatur, Illinois, and its associated distribution system was studied. Yeasts and acid-fast organisms were detected in all raw water samples, as were total coliform organisms. The density of yeasts averaged 5.7/ℓ in raw water samples and 1.5/ℓ in finished (treated) water. Organism densities of acid-fast bacteria averaged 37.0/ℓ in raw water samples and 2.2/ℓ in finished water. The proposed indicators were recovered from finished water samples with a frequency of 7 percent for yeasts and 20 percent for acid-fast organisms. Estimated standard plate counts were also performed and were found to be approximately 2×10^4/mℓ in raw water samples and approximately 7×10^3/mℓ in finished water samples. No significant correlation was found between the results of the standard plate count and the density of the other organisms determined, i.e., coliforms and the proposed indicator organisms.

Continuous flow chlorination, using mixed pure-cultures and diluted secondary wastewater effluent, confirmed the increased resistance of the proposed indicator organisms to free and combined chlorine, as compared to *E. coli* or coliforms. Experiments performed at pH 7 and 10 confirmed that greater yeast and acid-fast inactivation rates were achieved at the lower pH, thus supporting the claim that hypochlorous acid is a more effective disinfectant than hypochlorite ion. The relative resistance of these organisms to chlorination was found to be: acid-fast > yeast > coliforms.

Similar experiments were performed under breakpoint chlorination conditions. It was again observed that acid-fast organisms were the most resistant of the three organism groups studied, as evidenced by their ability to occasionally survive the severe conditions of breakpoint chlorination.

A step-wise regression analysis of the data from these studies showed that the independent variable of chlorine residual could explain greater than 85 percent of the variance in the inactivation of each of the test organisms.

Experiments were performed to study the reductions in the density of the proposed indicator organisms using clarified activated sludge effluent which had been passed through a granular activated carbon column. Yeast organisms were reduced approximately 8 percent, while coliforms and acid-fast organisms were each reduced by approximately 4 percent.

Laboratory coagulation/flocculation studies using alum or ferric chloride as the coagulant showed that removal of acid-fast and yeast organisms was similar to that for coliforms. In using lime, removal of yeasts was less than that observed for total coliforms. Studies on sand filtration indicated that acid-fast organisms were removed to the same extent as the coliforms, whereas yeasts showed a higher degree of removal than the coliforms. By optimizing coagulation conditions for turbidity removal, it was found that total coliforms, yeasts, and acid-fast organisms could be removed from inoculated water in the range of 90-99 percent. The percent removal of these organisms by sand filtration ranged from 40-70 percent for acid-fast and total coliform organisms, and was greater than 90 percent for yeasts.

Studies of organism inactivation by ozone were performed with acid-fast and yeast organisms, *E. coli*, *S. typhimurium*, and poliovirus type 1 (Mahoney).

Various parameters were analyzed to determine the extent of their influence on ozonation efficiency. Experiments performed with C. *parapsilosis* and M. *fortuitum* showed that the presence of ozone bubbles in addition to ozone residual was more effective in inactivating the test organisms than ozone residual alone. Ozone bubbles alone, without any ozone residual, were also found to cause a slight amount of inactivation. In a study on the effect of mixing rate in the ozone contact chamber, it was found that as the rate of agitation increased there was a higher decomposition rate of ozone and, consequently, a lower organism inactivation. Variations in pH did not significantly affect percent organism survival under constant ozone residuals. Experiments to study the effect of temperature indicated that the degree of inactivation of M. *fortuitum* increased significantly with an increase in temperature, at constant ozone residual. The relative resistance of test organisms to disinfection with ozone was found to be: M. *fortuitum* > poliovirus > C. *parapsilosis* > E. *coli* > S. *typhimurium*.

Studies performed to determine the mechanism of organism inactivation due to the action of free available chlorine demonstrated that the primary mode of action was cellular penetration at the level of the outer cell wall. This was additionally followed by disruption of cell permeability, secondary metabolic disturbances, and lethal lesions to the deoxyribonucleic acid (DNA). It was concluded that the increased resistance of C. *parpasilosis* and M. *fortuitum* to free available chlorine was a result of their cellular physiology, principally the thickness and rigidity, and impermeability, respectively, of their cell walls.

Information to date concerning the resistance of the proposed new indicator organisms, yeasts and acid-fast bacteria, to various forms of chlorine and the distribution of these organisms in water and wastewater indicates their potential use as indicators of chlorination efficiency.

SECTION 3

RECOMMENDATIONS

1. A large scale field sampling program to determine the densities of acid-fast and yeast organisms, as well as coliforms, and chlorine resistant pathogens, e.g., amoebic cysts, enteric viruses and bacteria, is required in order to develop criteria of disinfection efficiency based upon the proposed new indicator organisms with respect to the desired degree of microbial purity of a finished water supply or a treated wastewater effluent.

2. Providing the proposed indicator organisms continue to appear useful as a means of determining disinfection efficiency, the selective growth medium for the enumeration of acid-fast organisms and yeasts should be reexamined with the objective of reducing the incubation time required, or alternatively, rapid enumeration techniques should be investigated which enable the determination of the indicator organism densities within 24 hr.

3. If either the yeast or acid-fast group of organisms is accepted as a new indicator of disinfection efficiency, studies to determine the species of organisms most commonly found in water and wastewater should be performed to better define the nature of the indicator group.

SECTION 4

MATERIALS AND METHODS

INTRODUCTION

This section covers the biological, physical, and chemical techniques used in both the field and laboratory experiments related to the presence of the proposed indicator organisms and their role as indicators of disinfection efficiency by chlorine and ozone. Also included in this section are the methods of preparing the pure cultures of the yeasts and acid-fast organisms used in the laboratory experimental studies. The enumeration techniques for acid-fast organisms, yeasts, fecal coliforms, *E. coli*, *S. typhimurium*, and poliovirus have been reported in detail in earlier publications (5,6). In this section, modifications in the selective methods made since last reported shall be discussed.

SOURCE OF TEST ORGANISMS

The *M. fortuitum*, *M. phlei*, and *C. parapsilosis* test organisms used in this study were isolated from wastewater by Engelbrecht *et al.*(4). Poliovirus type 1 (Mahoney) and the cell line used (African Green Monkey Kidney [BGM] cells) were obtained from Dr. Gerald Berg, U.S. EPA, Cincinnati, Ohio. The pure cultures of *S. typhimurium* and *E. coli* were from the collection of the Department of Microbiology, University of Illinois at Urbana-Champaign.

ORGANISM ENUMERATION TECHNIQUES

Total Plate Count, Total Coliforms, Fecal Coliforms, *E. coli*, and *S. typhimurium*

The methodology used for the enumeration of total coliforms, fecal coliforms, *E. coli*, and *S. typhimurium* has been previously described (5). The determination of total plate count bacteria was performed according to Standard Methods (7).

Poliovirus

The enumeration method used for poliovirus has been described in detail (5).

Yeast

Depending on whether field or laboratory samples, yeasts were enumerated according to the procedures described earlier (5).

Acid-Fast Organisms

The methodology for enumerating acid-fast organisms in pure culture studies (laboratory studies) has been described (5,6). The standard enumeration technique for acid-fast organisms, used with mixed cultures or field samples as reported earlier, consisted of pretreating samples with 2.5 percent oxalic acid in a 1:1 volume ratio for 10 min and neutralizing with 2 percent sodium hydroxide (5,6). The samples were then membrane-filtered, with membranes being placed on Middlebrook 7H9 broth base (BBL, Cockeysville, MD) enriched with oleic acid-albumin-dextrose-catalase (OADC; Difco Laboratories, Detroit, MI), propionate, and antibiotics in a culture dish and incubated at 37°C for 72 hr. The filters were then removed from the agar surface, heat dried, and stained by means of the Brook's acid-fast stain. Colonies which appeared pink to red when viewed under a dissecting microscope were counted as acid-fast organisms.

Studies were performed in an attempt to simplify the pretreatment steps required in the enumeration of acid-fast organisms as described above. Two modifications were evaluated using both a pure culture of M. *fortuitum* suspended in pH 7 buffered deionized-distilled water and the natural population of acid-fast organisms occurring in raw wastewater, diluted with pH 7 buffered deionized-distilled water.

Modification 1. The untreated sample was filtered and an equal volume of 2.5 percent oxalic acid was added and allowed to remain on the filter by disconnecting the vacuum. After 10 min, the oxalic acid was filtered and the membrane filter rinsed with pH 7 buffer.

Modification 2. The sample was treated with 2.5 percent oxalic acid (1:1 volume ratio) in a flask and then filtered after 10 min contact time, without sodium hydroxide neutralization.

Results of these modified procedures are presented in Tables 1 and 2. As indicated, no statistically significant difference among the results obtained by use of the three treatments was observed for either the wastewater or the pure culture suspended in buffered deionized-distilled water. Figure 1 is a diagram of the revised enumeration procedure for acid-fast organisms.

An investigation was also performed in an attempt to more easily process large volume samples containing low organism densities by increasing the concentration of oxalic acid stock solution, with a parallel decrease in the volume of the filtrate produced. Results of these experiments are presented in Table 3 and indicate a statistically significant difference in

TABLE 1. ACID-FAST PRETREATMENT METHODOLOGY, SAMPLE: *M. fortuitum* PURE CULTURE IN BUFFERED DEIONIZED-DISTILLED WATER

RAW DATA

	Counts per 100 mℓ sample		
	Pretreated on filter - NaOH omitted	Pretreatment in flask - NaOH omitted	Standard Pretreatment
Replicates	78, 85, 62, 59, 91, 71	78, 108, 77, 65, 62, 84	76, 83, 78, 69, 93, 56
Mean	74.3	79.0	75.8
Standard Deviation	12.7	16.5	12.6

ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F
Among Treatments	2	68.11	34.06	0.17*
Within Treatments	15	2950.19	196.68	
Total	17	3018.3		

*Not significant ($p > 0.05$)

counts/100 mℓ with concentrations of oxalic acid greater than 2.5 percent. It may also be noted that the average organism recovery decreased with an increasing concentration of oxalic acid.

The standard pretreatment technique, described earlier, with neutralization by sodium hydroxide was used to study the frequency of occurrence of acid-fast organisms at the St. Joseph wastewater and Oakwood water treatment plants and in the Salt Fork of the Vermillion River as well as in the coagulation-flocculation and sand filtration studies. Pretreatment Modification 2 was used in the distribution study at Decatur in the continuous flow chlorination study. The pretreatment step and the addition of antibiotics and selective agents to the medium was omitted in performing the studies related to mechanism of inactivation of acid-fast organisms by chlorine.

PREPARATION OF PURE CULTURES (Laboratory Studies)

Acid-Fast Organisms

Acid-fast organisms (*M. fortuitum* and *M. phlei*) were grown in

TABLE 2. ACID-FAST PRETREATMENT METHODOLOGY,
SAMPLE: 0.5% RAW WASTEWATER

RAW DATA			
	Counts per 100 mℓ sample		
	Pretreated on filter - NaOH omitted	Pretreatment in flask - NaOH omitted	Standard pretreatment
Replicates	163, 144, 154, 128, 154	190, 188, 165, 176, 158, 158	138, 157, 166, 199, 150, 156
Mean	148.6	172.5	161.0
Standard Deviation	13.3	14.4	20.8

ANALYSIS OF VARIANCE				
Source of variation	Degrees of freedom	Sum of squares	Mean squares	F
Among Treatments	2	1559.42	779.71	2.79*
Within Treatments	14	3906.7	279.05	
Total	16	5466.12		

*Not significant ($p > 0.05$)

Middlebrook 7H9 medium for 36 hr at 37°C in a water shaker bath and then centrifuged in a Sorval GLC-2 (DuPont Instruments, Newton, CT) general laboratory centrifuge at 1800 rpm for 15 min. In the ozone studies, the incubation time was 72 hr. The pellets were then washed twice with a total of at least 150 mℓ of pH 7 phosphate buffer. Cells prepared in this manner were resuspended in phosphate buffer and were kept at 4°C until needed. Before the organisms were used, the optical density was measured at 660 nm using cuvetts with a 1 cm light path in a Bausch-Lomb Spectronic 20 colorimeter (Bausch-Lomb, Inc., Rochester, NY). The optical density was correlated with a previously developed extinction coefficient. The concentration of cells was calculated as the ratio of the optical density to the extinction coefficient, times the length of the light path.

Yeast

The yeast culture (C. *parapsilosis*) was grown in modified yeast extract-malt extract medium for 24 hr at room temperature on a shaker. In the case of the ozone studies, incubation was for 48 hr. Centrifugation, washing, and subsequent determination of organism density was performed by the same procedure as described for acid-fast organisms.

Sample

↓

Homogenization
Waring Blender (30 sec)
or agitation

↓

Pretreatment*
1.25% Oxalic Acid (10 min)

↓

Dilution
Phosphate Buffer, pH 7

↓

Membrane Filtration

↓

Cultivation
7H9 Middlebrook
(plus OADC, propionate, and antibiotics)

↓

Incubation
72 hr - 37°C

↓

Staining*
Heat fix colonies on membrane
Brook's carbol fuchsin (primary stain)
10% oxalic acid rinse
Brook's malachite green (counterstain)
Distilled water rinse

↓

Enumeration
Dark pink to red colonies

*These steps were omitted in the laboratory studies using pure acid-fast cultures.

Figure 1. Revised Enumeration Technique for Acid-Fast Organisms.

TABLE 3. ACID-FAST PRETREATMENT METHODOLOGY USING DIFFERENT CONCENTRATIONS OF OXALIC ACID, SAMPLE: 0.5% RAW WASTEWATER

RAW DATA

	Counts per 100 mℓ sample		
	Pretreated with 10% oxalic acid	Pretreated with 5% oxalic acid	Standard pretreatment 2.5% oxalic acid
Replicates	129, 108, 64, 71, 94, 106, 95, 97, 83, 59	102, 48, 45, 122, 96, 56, 137, 149, 49, 108	175, 125, 159, 97, 118, 89, 127, 117, 95, 117
Mean	90.6	109.2	121.9
Standard Deviation	21.69	26.03	27.33

ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F
Among Treatments	2	4,956.47	2,478.24	3.924*
Within Treatments	27	17,052.90		
Total	29	22,009.37	631.59	

*Significant ($p > 0.05$)

Escherichia Coli

Preparation of *E. coli* suspensions was performed by inoculating a young culture of the organism into a flask containing nutrient broth and incubating overnight at 37°C in a shaker water bath. Centrifugation, washing, and determination of cell density was carried out by the same procedure as that for acid-fast organisms.

Salmonella typhimurium

The method followed for preparation of *S. typhimurium* was the same as that for E. *coli*. However, the incubation period was 48 hr.

Poliovirus

Poliovirus type 1 (Mahoney) was grown on monolayers of BGM cells. Infected cells, along with the suspending medium, were frozen at -70°C and thawed twice, then centrifuged for 1 hr at 10,000 rpm in a Beckman L2-65B ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The supernate was decanted and recentrifuged at 50,000 rpm for 2 hr. The pellet from the second centrifugation, containing the virus, was resuspended in 10 to 20 mℓ of buffered deionized water and kept overnight at 4°C, after which the virus suspension was again centrifuged at 50,000 rpm for 2 hr. The virus was finally resuspended in 150 mℓ of phosphate buffer distributed in small aliquots, and then frozen and stored at -70°C for use in subsequent experiments. The virus was thawed and diluted before use.

PHYSICAL AND CHEMICAL METHODS

Temperature

Temperature was recorded by mercury-filled centrigrade thermometers in laboratory experiments and by a temperature measuring probe attached to a YSI Model 54 DO analyzer (Yellow Springs Instrument Co., Yellow Springs, OH) in the field studies.

pH

The electrometric method, using glass and reference electrodes with a Beckman Electromate pH meter (Beckman Instruments, Inc., Palo Alto, CA), was used to measure pH.

Dissolved Oxygen

Dissolved oxygen was measured by means of a YSI Model 54 DO meter (Yellow Springs Instrument Co., Yellow Springs, OH).

Total Suspended Solids

Total suspended solids were determined according to the procedure stated in Standard Methods using glass fiber filter discs (7).

Total Organic Carbon and Chemical Oxygen Demand

Total organic carbon (TOC) measurements were made with a Beckman Model 915 TOC analyzer (Beckman Instruments, Inc., Palo Alto, CA) using the two-channel method, as given in Standard Methods (7). Chemical oxygen demand (COD) was measured according to the procedure given in Standard Methods (7).

Turbidity

Turbidity was measured in a Hach Turbidimeter Model 2100A (Hach Chemical Co., Ames, IA) and reported in nephelometric turbidity units (NTU).

Chlorine Residuals

Chlorine residuals were measured by the N,N-diethyl-p-phenylene-diamine (DPD) method in accordance with Standard Methods (7) for the batch chlorination experiments and field studies. The measurement of chlorine residuals in the continuous chlorination and in the mechanism of inactivation studies was determined by the amperometric method as described in Standard Methods (7) using an amperometric titrator (Series A-790, Wallace and Tiernan, Belleville, NJ).

Ozone Residual

Ozone residual in the aqueous phase was determined by using the UV spectrophotometric method of Shechter (8).

Chlorine-Demand Free Buffer

Chlorine-demand free buffer (CDFB) was prepared as described in a previous report (5). Deionized-distilled water was chlorinated by adding sodium hypochlorite to achieve a free chlorine residual of approximately 3 mg/ℓ, and stored at room temperature for one week. The water was then divided into 5 ℓ portions and the appropriate quantity of phosphate or borate salt was added to each portion to achieve the desired pH. Each portion was subsequently boiled for sterilization and then placed under UV light for at least 72 hr for final dechlorination. The CDFB was then ready for use.

FIELD METHODS

Occurrence of Indicator Organisms in Water and Wastewater

A detailed field survey of the densities of acid-fast organisms, yeasts, fecal coliforms, and total coliforms was undertaken. The location selected for this survey was the St. Joseph, Illinois, wastewater treatment plant, its receiving stream, the Salt Fork of the Vermillion River, and the Oakwood, Illinois, water treatment plant, which uses the Salt Fork as a water supply.

The wastewater treatment plant at St. Joseph, Illinois, serves a population of 1850. A combined sewer system carries domestic wastewater and storm runoff to the treatment plant. A schematic of the plant is given in Figure 2. Raw wastewater flows into an inlet chamber for removal of grit and is then fed into two Model R Oxigest Tanks (Smith and Loveless Co., Lenexa, KS) which are operated on the contact stabilization principle. Waste sludge is aerobically digested and then lagooned. The secondary effluent is chlorinated prior to discharge into the Salt Fork of the Vermillion River. The operating characteristics of the treatment plant for the period March 1975 to January 1976 are summarized in Table 4.

As noted in Figure 2, there were three sampling locations within the treatment plant. SJ1 was the de-gritted wastewater immediately upstream from the aerator influent. SJ2 was the activated sludge effluent prior to chlorination, and SJ3 was the chlorinated effluent immediately prior to discharge to the receiving stream. Grab samples were used for analysis and samples were taken without compensating for hydraulic detention time.

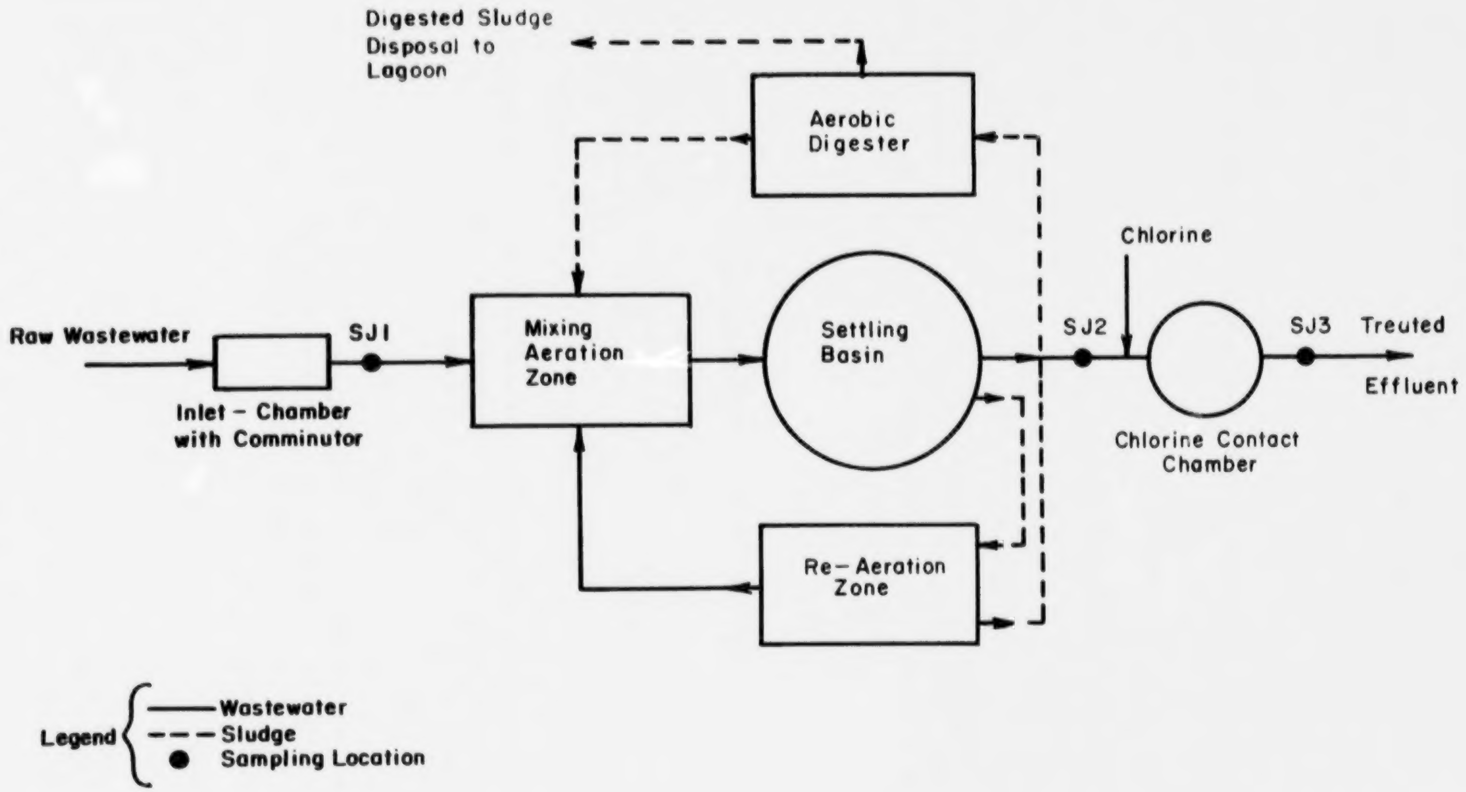
Digested Sludge
Disposal to
Lagoon
Aerobic
Digester
Chlorine
Raw Wastewater
SJ1
Mixing
Aeration
Zone
Settling
Basin
SJ2
SJ3
Treuted
Effluent
Inlet - Chamber
with Comminutor
Chlorine Contact
Chamber
Re-Aeration
Zone
Legend
Wastewater
Sludge
Sampling Location

Figure 2. St. Joseph, Illinois, Wastewater Treatment Plant

TABLE 4. OPERATING CHARACTERISTICS OF THE ST. JOSEPH, ILLINOIS WASTEWATER TREATMENT PLANT (March 1975 - January 1976)

Parameter	Range	Mean
Flow, m^3/day		
Design capacity	-	1135
Actual	-	662
BOD_5, mg/ℓ		
Influent	310-460	380
Effluent	3-9	5
Suspended Solids, mg/ℓ		
Influent	330-560	400
Effluent	2-6	4
Chlorine, mg/ℓ		
Dosage	3.5-4	-
Total Residual	0.6-1.1	0.8

The Oakwood, Illinois, water treatment plant serves a population of 1600. A schematic diagram of the plant is shown in Figure 3. Raw water is pumped from the Salt Fork of the Vermillion River, at a point approximately 59.5 km downstream from the St. Joseph wastewater treatment plant discharge, into a 5677.5 m^3 reservoir adjacent to the treatment plant as it is needed. A typical turnover time in the reservoir is two weeks; however, short circuiting may occur. During summer months, copper sulfate is added to the reservoir to control algae, at a dose sufficient to produce a final copper concentration of approximately 1 mg/ℓ. Operating characteristics of the plant are shown in Table 5.

As noted in Figure 3, four sampling locations within the treatment plant were used. OW1 was located at the head end of the raw water reservoir. The OW2 sample was taken from the overflow of the clarifiers. OW3 was located immediately after sand filtration, and the OW4 sample, the finished water, was taken at a service tap in the treatment plant.

The Salt Fork of the Vermillion River was sampled at locations between the U.S. Highway 150 bridge, upstream of the St. Joseph wastewater plant, and the Oakwood water treatment plant intake. The approximate locations of the sampling stations are noted in Figure 4. Table 6 gives an exact description of the sampling sites used. It should be noted in Table 6 that sample station RSO is located above the outfall of the St. Joseph wastewater treatment plant, whereas the remaining five sample stations are all located below the outfall.

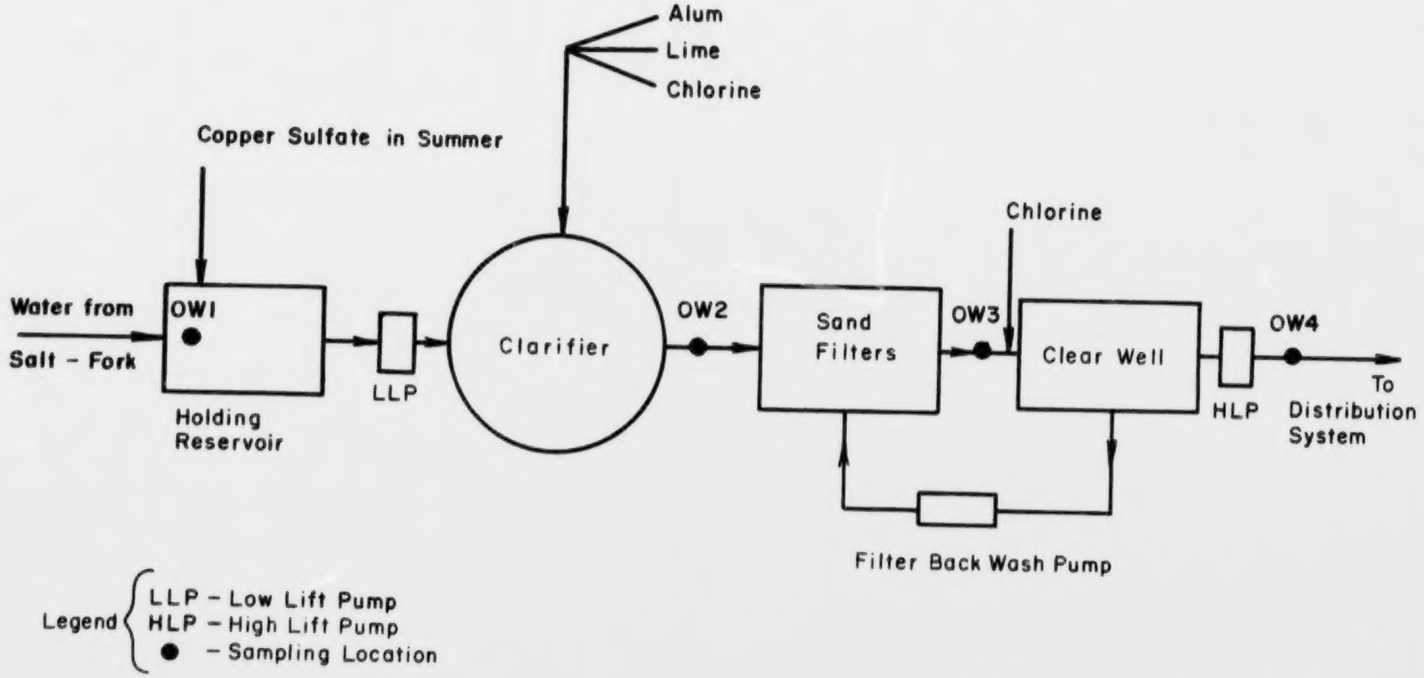
Alum
Lime
Chlorine
Copper Sulfate in Summer
Chlorine
Water from
Salt - Fork
OW1
Holding
Reservoir
LLP
Clarifier
OW2
Sand
Filters
OW3
Clear Well
HLP
OW4
To
Distribution
System
Filter Back Wash Pump
Legend
LLP - Low Lift Pump
HLP - High Lift Pump
● - Sampling Location

Figure 3. Oakwood, Illinois, Water Treatment Plant

TABLE 5. OPERATING CHARACTERISTICS OF THE OAKWOOD, ILLINOIS, WATER TREATMENT PLANT (March 1975 - January 1976)

Parameter	Range	Mean
Design Flow Capacity, m^3/day		545.2
Actual Flow, m^3/day		321.8
Alum Dosage, kg/day		13.6
Lime Dosage, kg/day		36.3
Filtration Rate, ℓ/min/m^2		101.8
Length of Filter Run, days		2
Terminal Chlorination Dosage, mg/ℓ		7.0
Finished Water		
Total Hardness, mg/ℓ	180-270	220
Total Alkalinity, mg/ℓ	100-180	150
pH	7.0-8.6	7.8
Turbidity, NTU	1-9	2.0
Total Residual Chlorine, mg/ℓ	0.6-1.5	1.0

The Salt Fork of the Vermillion arises in east Champaign County, Illinois, at an altitude of 243.8 m above sea level. It flows toward the southeast and joins the Middle Fork of the Vermillion River southwest of Danville, Vermillion County, Illinois. The Vermillion River joins the Wabash River near the Illinois-Indiana state line (9).

The discharge from the city of Rantoul municipal wastewater treatment plant enters the Upper Salt Fork drainage ditch. Wastewater discharge from the Chanute Air Force Base enters the same drainage ditch approximately 3.2 km below the Rantoul outfall. Effluent from the East Side Treatment Plant of the Urbana-Champaign Sanitary District, and urban runoff from the cities of Urbana and Champaign enter the Saline Branch on the northeast edge of the city of Urbana. The Saline Branch subsequently joins the Upper Salt Fork approximately 1.6 km west of the village of St. Joseph to form the Salt Fork of the Vermillion River. Wastewater effluent from the St. Joseph wastewater treatment plant is discharged into the Salt Fork approximately 1.6 km

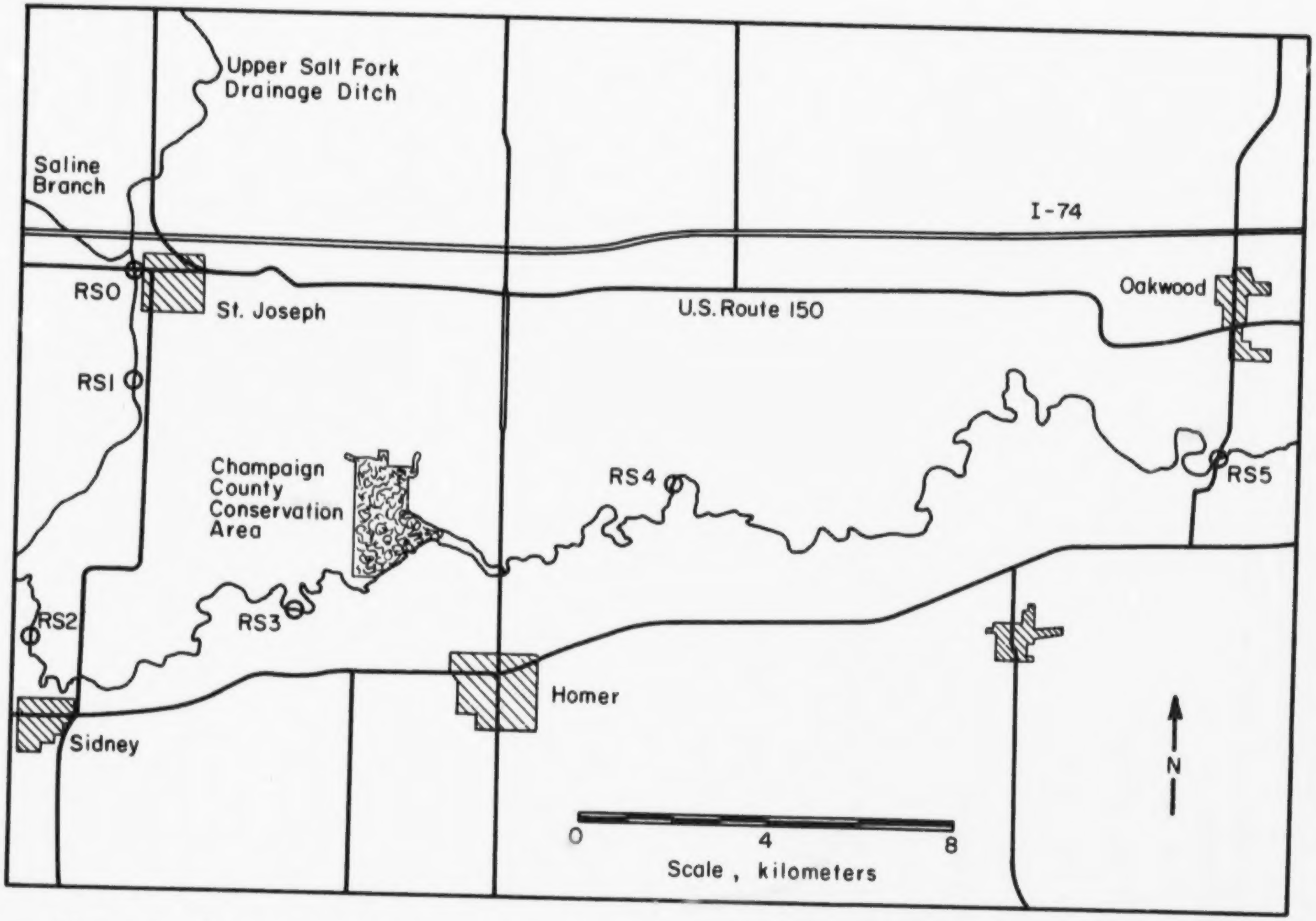
Upper Salt Fork
Drainage Ditch
Saline
Branch
I-74
RSO
St. Joseph
U.S. Route 150
Oakwood
RS1
Champaign
County
Conservation
Area
RS4
RS5
RS2
RS3
Homer
Sidney
N
0
4
8
Scale , kilometers

Figure 4. Salt Fork of the Vermillion River and Sampling Stations

TABLE 6. DETAILED DESCRIPTION OF THE SALT FORK OF THE VERMILLION RIVER SAMPLING STATIONS

Site designation	Description
RS0	U.S. Highway 150 bridge over the Salt Fork, west of St. Joseph
RS1	Bridge over the Salt Fork, 2.4 km south of St. Joseph, approximately 88° 03' 00" and 40° 05' 30"
RS2	Bridge over the Salt Fork, 1.6 km north of Sidney, Illinois, approximately 88° 04' 30" and 40° 02' 20"
RS3	Bridge over the Salt Fork, 4.8 km west of Homer, Illinois, approximately 88° 00' 30" and 40° 02' 50"
RS4	Bridge over the Salt Fork, 4.8 km northeast of Homer, approximately 87° 54' 30" and 40° 04' 20"
RS5	Bridge over the Salt Fork, 3.2 km south of Oakwood, on Federally Aided Secondary Highway 331

south of the confluence of the Saline Branch and the Upper Salt Fork. Other than field tile drainage nets, which are common in the east central Illinois agricultural area, there are no other known continuous discharges into the Salt Fork between St. Joseph and Oakwood. However, diffuse sources of wastewater, largely untreated, may enter this stretch of the stream from other communities located within the drainage basin.

The sampling program was initiated in March, 1975. Two sets of samples, collected in March and April, 1975, were used to establish the sampling procedures and protocol. River stations RS1 and RS5 were not included in these two sets of samples. Data for the first two sets have not been included in the statistical analyses.

Initially, a set of samples was collected and analyzed twice monthly. After reviewing data of the first four sets, it was decided to reduce sampling frequency to monthly. No sampling could be performed during December 1975 due to the extremely poor weather conditions; however, two sets of samples were collected in January 1976. This report considers the data obtained during the period May 1975 through January 1976. Eleven sets of samples were collected and analyzed during this nine-month period.

All stream water samples for microbiological analysis were collected 5-10 cm below the water surface in sterile 1 ℓ plastic bottles; usually the sample was collected by lowering the freshly opened bottle from a bridge.

Samples were collected from the Oakwood and St. Joseph treatment plants from the points previously described. All samples were dechlorinated by the addition of 1 mℓ of 0.05 N sodium thiosulfate per liter of sample immediately after collection.

Sample collection was initiated at RS0 between 0900 and 1000 hr; samples were then collected at the St. Joseph wastewater treatment plant, i.e., RS1-RS5, and finally at the Oakwood water treatment plant. Immediately after collection, the dechlorinated samples were stored in an ice chest. Samples were transported back to the laboratory by 1200 hr, and it was generally possible to complete the analyses by 1700 hr.

During the 24-hr field study conducted at the St. Joseph wastewater treatment plant, samples were collected at 1200, 1500, 1700, 1900, 2200, 0100, 0400, 0700, and 1200 hr. Predetermined dilutions of the samples were membrane-filtered on site, and placed on the appropriate agar medium and refrigerated; the prepared samples were then transported back to the laboratory for incubation and enumeration.

Occurrence of Indicator Organisms in Distribution Systems

Another field survey was performed at Decatur, Illinois, South Side Water Treatment Plant between February 8 and September 11, 1977. This survey involved the enumeration of acid-fast, yeast, and total coliform organisms as well as the standard plate count in the raw water, in the finished water at the plant following treatment, and in treated water at various locations in the distribution system. A total of 18 sets of samples were collected during the study period; however, this report summarizes data from only the last 14 sets of samples. The first four sets were used to refine and modify the laboratory procedures for processing of the samples.

The South Side Plant is one of two water treatment plants serving a population of 95,000 in the city of Decatur. Water is withdrawn from Lake Decatur, a man-made impoundment on the Sangamon River. Treatment consists of alum coagulation-flocculation, sand filtration, and both pre- and post-chlorination. The water is softened by only a small degree. Plant operating data for the South Side Plant for the period April 1977 through October 1977 are summarized in Table 7.

A major distirubtion line extends for approximately 6.4 km to the west of the South Side Water Treatment Plant. This water main was selected for sampling purposes because it is supplied by water treated almost exclusively by the South Side Plant, and because it terminates in a deadend. The North Side Water Treatment Plant distribution network does interconnect with the previously described water main in the vicinity of sample station 4, and this may have resulted in a partial mixing of water from each treatment plant in samples taken from stations 4 and 5. A schematic map showing the location of sampling points is given in Figure 5, and a more detailed description of each sample station is given in Table 8.

TABLE 7. OPERATING DATA FOR THE SOUTH SIDE WATER TREATMENT PLANT, DECATUR, ILLINOIS

Parameter	Average value
Flow, m^3/day	
Design capacity	113,550
Actual	56,775
Turbidity, NTU	
Raw water	21
Pre-filtration	6.3
At plant tap (finished water)	0.6
Temperature, °C	
Raw	69
At plant tap (finished water)	76
pH	
Raw	8.0
At plant tap (finished water)	8.5

TABLE 8. LOCATION OF SAMPLING STATIONS, DECATUR, ILLINOIS

Sampling Station	Location
1	Raw water sampled at the South Side Plant
2	Finished water sampled at a service tap of the South Side Plant
3	Fire station near Main and Franklin Streets
4	Bowling alley at Fairview Avenue and Eldorado Streets
5	Holiday Inn on Eldorado Street

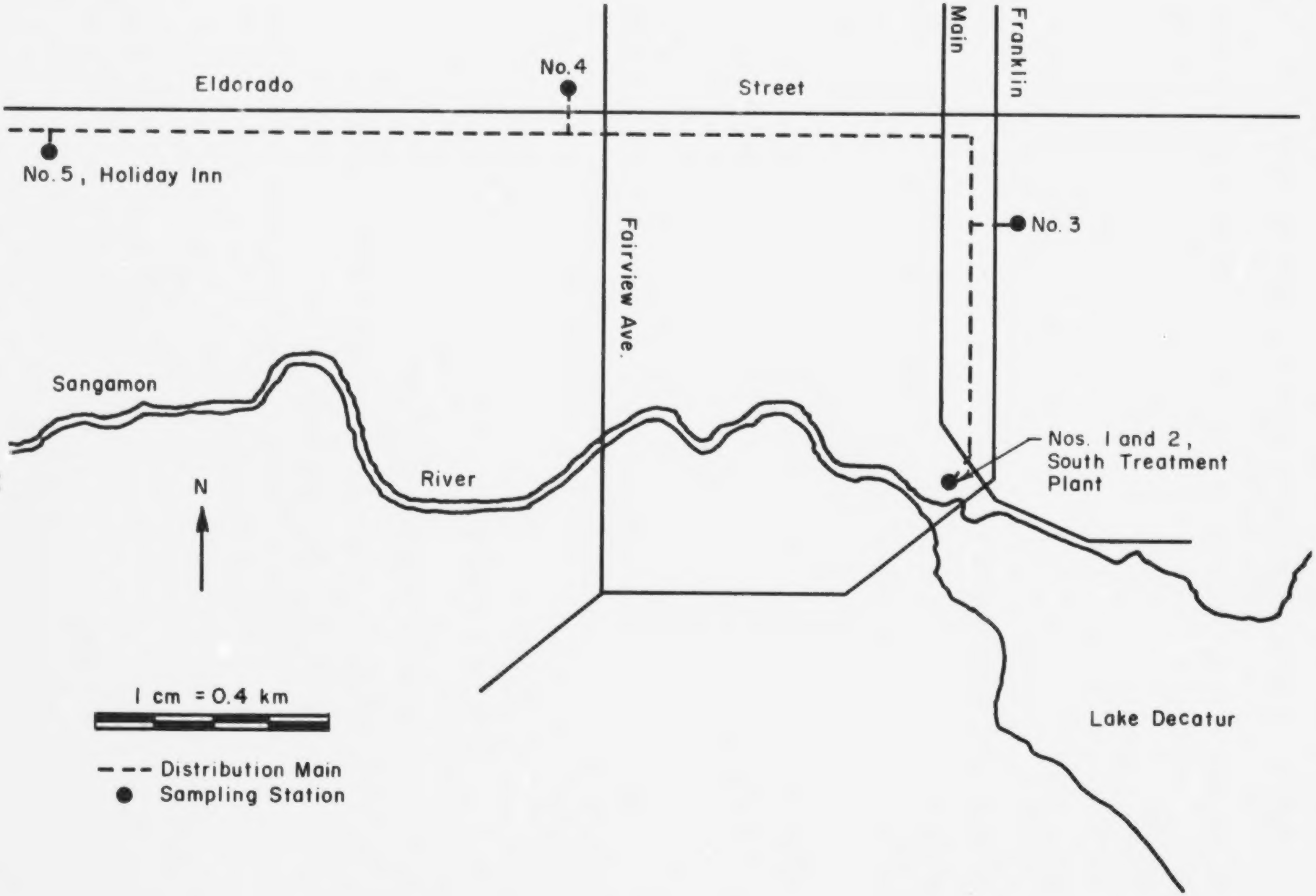
Eldorado
Street
No. 4
Main
Franklin
No. 5, Holiday Inn
Fairview Ave.
No. 3
Sangamon
River
Nos. 1 and 2, South Treatment Plant
N
Lake Decatur
1 cm = 0.4 km
--- Distribution Main
● Sampling Station

Figure 5. Water Treatment Plant and Distribution System with Sampling Stations, Decatur, Illinois

A sample of approximately 8 ℓ was taken at each station by personnel of the city of Decatur and immediately dechlorinated with sodium thiosulfate. At the same time, a field determination of free available and total chlorine residual was made using the modified DPD method (LaMotte Chemical, Chestertown, MD). The samples were then transported to the laboratory for further analysis. All analyses were completed within 8 hr of the time of sample collection.

Total coliform organisms were enumerated by both membrane filtration (MF) and most probable number (MPN) techniques (7). With the MF technique, raw water samples were analyzed using triplicate filtrations of three different dilutions, selected so as to yield between 20 and 80 colonies per plate. Treated water was uniformly filtered in duplicate 100 mℓ volumes. Colony counts were geometrically averaged and reported as "number/100 mℓ." Analysis of the water for coliforms by the MPN method consisted of five tubes for each of three different dilutions. Treated water was analyzed using a 5-tube, single-dilution design. Coliform densities have been reported as "MPN index/100 mℓ," using the MPN tables in Standard Methods (3).

Acid-fast organisms and yeasts were enumerated using the techniques previously described (5). Duplicate 1-ℓ sample volumes from each station were membrane filtered; colony counts at each station were geometrically averaged and reported as "number/ℓ."

Standard plate count enumeration was performed on every sample using duplicate plates for each of three different dilutions. Colonies were counted on a Darkfield Quebec Colony Counter (Fischer Scientific, Pittsburgh, PA), and the "estimated standard plate count/mℓ" was calculated as described in Standard Methods (7).

UNIT OPERATIONS/UNIT PROCESSES LABORATORY METHODS

Continuous Flow Chlorination Experiments

Continuous flow experiments were designed to determine the chlorine resistance of the proposed indicator organisms and coliform organisms under various idealized water and wastewater chlorination conditions. To simulate the conditions used in actual water and wastewater chlorination practice, a tubular reactor was fabricated using a plexiglass tube of approximately 3.9 cm internal diameter and 183.5 cm length. The overall theoretical detention time of the reactor was approximately 20 min for a flow rate of 100 mℓ/min. Additional sampling ports corresponding to detention times of 4, 8, 12, and 16 min were located along the longitudinal axis of the reactor. A schematic diagram of this reactor is shown in Figure 6. The experimental arrangement for all continuous flow experiments was such that the influent flow to the reactor was a combination of both the solution containing the test organisms and the free chlorine feed solution, with mixing of these two flows occurring just prior to their entry into the reactor. The total combined influent flow rate was controlled at approximately 100 mℓ/min, thus resulting in the reactor effluent detention time of approximately 20 min. To verify the plug flow

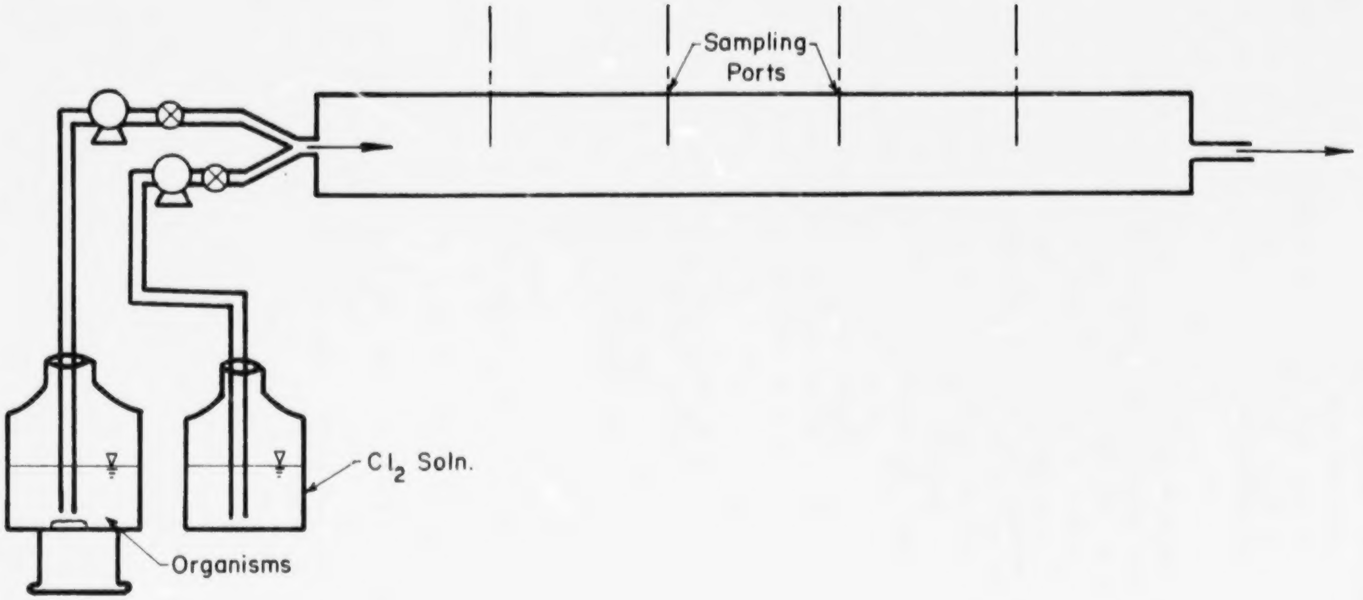
Sampling Ports
Cl_2 Soln.
Organisms

Figure 6. Continuous Flow Reactor for Chlorine Inactivation Studies

nature of the reactor, dye tracer studies were made. Figures 7 and 8 show typical results of these tracer studies. These figures show that plug-flow conditions prevailed in the reactor.

The continuous flow experiments performed using the above described reactor consisted of two basic types: 1) mixed pure culture inactivation studies, and 2) natural population inactivation studies. In the mixed pure culture chlorination studies, experimental conditions were established to assure the observation of several logs of organism inactivation, as well as a low chlorine demand with a reasonable free chlorine concentration. These studies were made with a laboratory-prepared "mixed culture" of the yeast, acid-fast, and coliform organisms. This mixed culture of test organisms was suspended in CDFB and fed to the reactor at a flow rate of approximately 25 mℓ/min, along with the free chlorine solution at a flow rate of approximately 75 mℓ/min. The reactor was allowed to flow full with the combined test solutions for a minimum of two reactor detention times before test samples were collected. Experimental test samples from the reactor, at all but the reactor effluent port, were collected by means of syringes inserted through the membrane-covered ports located along the reactor length so as to give the desired retention times of approximately 4, 8, 12, and 16 min. Samples having a retention time of 20 min were collected in sterile flasks by means of plastic tubing connected to the end of the reactor.

In all test samples collected, 0.05 N sodium thiosulfate was added immediately to destroy the chlorine residual. Culturing and enumeration techniques for the acid-fast, yeast and coliform organisms were performed as previously described for the laboratory studies (5). The amperometric method was used to measure the free chlorine residual of both the chlorine feed solution and the reactor effluent.

The natural population studies were made using the clarified activated sludge effluent of the East Side Wastewater Treatment Plant of the Urbana-Champaign Sanitary District as the organism feed solution. The East Side Plant consists of primary treatment followed by parallel secondary treatment, i.e., activated sludge and trickling filter units, clarification and chlorination. It had previously been shown that acid-fast, yeast, and coliform organisms were routinely present in both the plant's raw influent and in the activated sludge effluent (5).

The sample of activated sludge effluent for each natural population experiment was collected at the point of overflow from the activated sludge clarifier to the chlorination contact tanks. A sufficient quantity of effluent, approximately 12 ℓ, was collected to allow each experiment to be completed using a single sample and to allow a physical and chemical analysis to be made. Each sample of effluent was analyzed for pH, temperature, TOC, suspended solids, turbidity, and occasionally COD.

These chlorination experiments were performed in a manner similar to the "mixed culture" studies with free chlorine, but several notable procedural changes were made. The influent flow to the reaction chamber was again approximately 100 mℓ/min with a resulting effluent detention time of

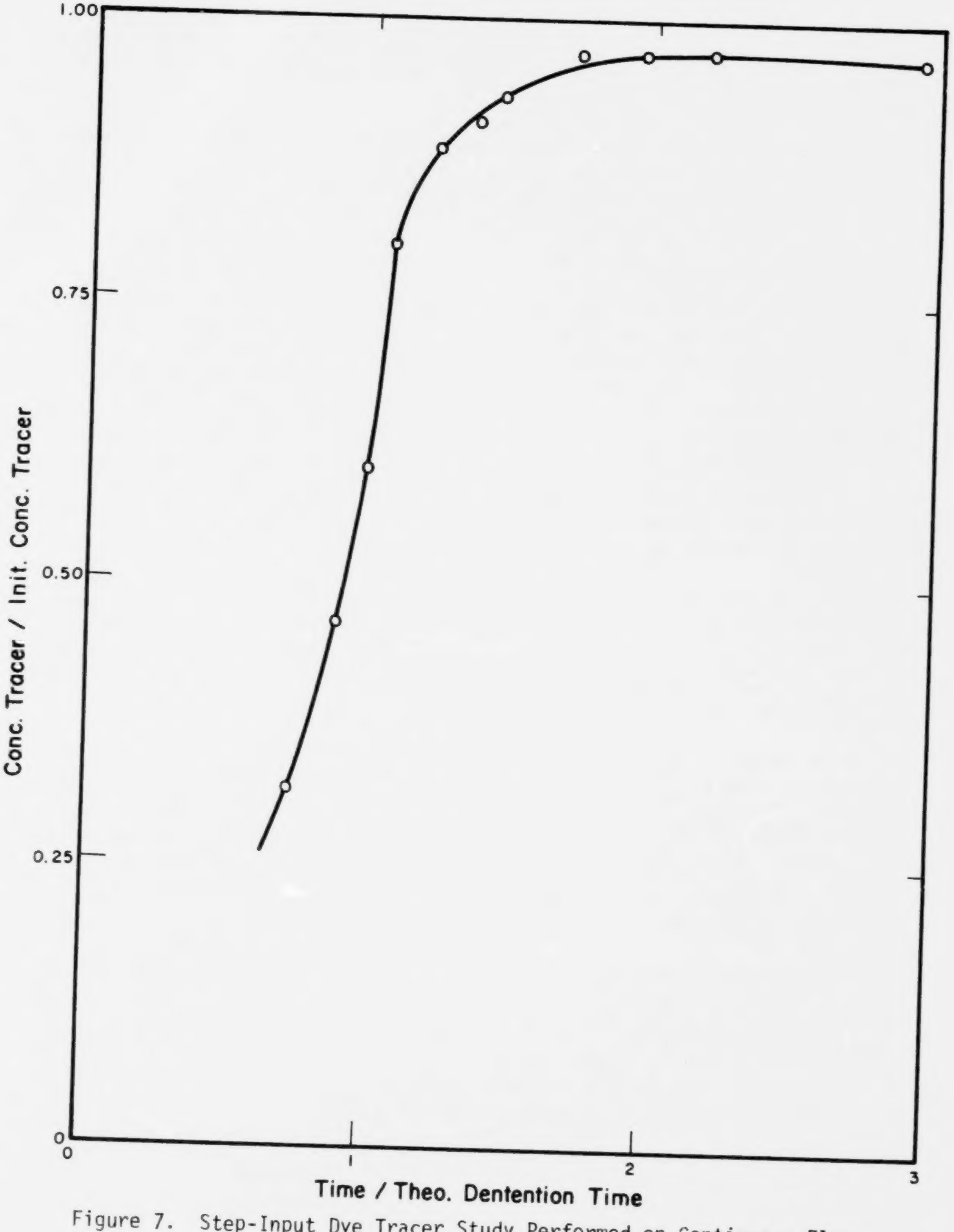

Figure 7. Step-Input Dye Tracer Study Performed on Continuous Flow Inactivation Reactor

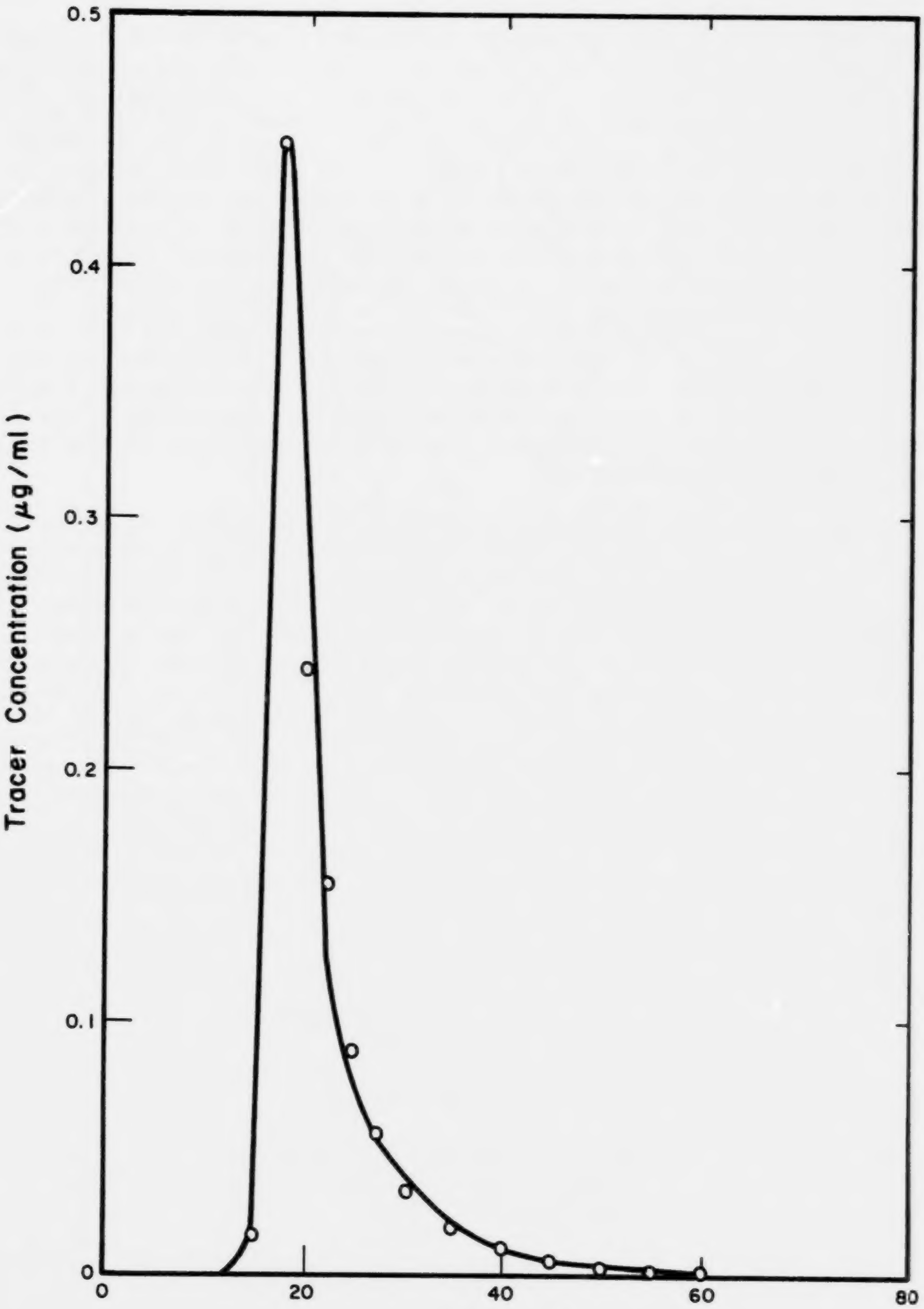

Figure 8. Pulse-Input Dye Tracer Study Performed on Continuous Flow Inactivation Reactor

approximately 20 min, but the reactor inflow was a combination of 90 mℓ/min of the sample feed solution (clarified activated sludge) and 10 mℓ/min of the free chlorine feed solution. This flow ratio of 9:1 resulted in an undesirable dilution of the sample to be disinfected, but was unavoidable due to the limits of the feed pumps used in the experiments. Due to the chlorine demand of the clarified activated sludge, it was found that whereas the chlorine feed solution was free available chlorine, the residual chlorine, as measured in the reactor effluent, was always present as combined available chlorine, except in the case of the breakpoint chlorination experiments. For the non-breakpoint experiments, a 20 min contact time was a sufficient reaction time to convert the free chlorine in solution to combined chlorine, and the chlorine residual was thus measured as total combined available chlorine. The breakpoint chlorination experiments involved measurement of free residual chlorine only. The natural population chlorination studies were performed without any adjustment of the pH or temperature of the activated sludge effluent feed solution; the chlorine feed solution was buffered at pH 7 and at room temperature.

In an attempt to investigate the inactivation behavior of the test organisms under conditions resembling advanced wastewater treatment and/or water reuse, chlorination experiments were performed utilizing an activated carbon treated secondary wastewater. All experiments were performed using the same activated carbon treated clarified activated sludge effluent. The activated carbon treatment consisted of passing the secondary wastewater effluent by upflow through a semi-expanded column of granular activated carbon (approximately 340 g, Grade 10 x 30, Darco Activated Carbon, ICI Americus Inc., Wilmington, DE). The theoretical column detention time was 8.5 min at a hydraulic loading of approximately 114 $\ell/\mathrm{min}/\mathrm{m}^2$ for the 3.8 cm inside diameter, tubular column reactor. The effluent from the activated carbon column was collected and used as the influent organism test solution to the chlorination reactor. The clarified activated sludge solution was passed through the carbon column for at least 8 hr at the described application rate before a sample was collected for the actual chlorination experiment; this permitted the carbon column to reach a steady state condition. The influent and effluent of the carbon column were characterized as to pH, turbidity, suspended solids, temperature and total organic carbon. The chlorination experiments followed the same experimental procedures as outlined for the other natural population studies.

Coagulation-Flocculation and Filtration Experiments

To determine the removal of yeasts and acid-fast organisms by various treatment processes, a series of laboratory studies were performed using coagulation-flocculation and sand filtration.

Coagulation-flocculation performance with respect to removing the test organisms was studied using typical jar-test procedures. Each series of experiments was designed to achieve the optimum removal of turbidity in the water tested. This was accomplished by optimizing the coagulant dosage, taking into account pH, flocculation time, and mixing speed. The jar tests were performed using six-bladed paddles and a Phipps and Bird multiple position flocculator (Phipps and Bird, Inc., Richmond, VA). Water samples were placed in 1 ℓ beakers and stirred for 2 min at 100 rpm after which sufficient 1.0 N HCl or 1.0 N NaOH was added for pH adjustment, when necessary, along with the appropriate dosage of coagulant. Following this, flash mixing at 100 rpm for 2 min was accomplished. The stirring rate was then reduced to the desired flocculation speed for a predetermined time. After 30 min of settling, the supernate was decanted and the turbidity measured. Samples were also removed for the enumeration of yeasts, acid-fast, and total coliform organisms.

Seven series of coagulation experiments were performed. Three of these used alum as a coagulant, and two each used ferric chloride and lime. One series with each coagulant was conducted using river water collected from the Salt Fork of the Vermillion River at Station RS1. The remaining series of experiments for each coagulant were performed using dechlorinated tap water to which a caolinite clay suspension and one percent raw municipal wastewater were added. The experiments with river water were made at 20°-25°C, while the dechlorinated tap water experiments were performed at 10°-15°C. The tap water had an alkalinity of 180 mg/ℓ as $CaCO_3$ and a pH of 7.9-8.2, while the river water typically had an alkalinity of 350 mg/ℓ and a pH of 7.1-7.2.

The stock suspension of kaolinite was prepared by adding 50 g kaolinite clay (Kaolinite #7, Ward's Natural Science Establishment, Rochester, NY) to 20 ℓ of tap water. After shaking, the suspension was allowed to settle for 1.5 hr. The suspension was decanted to remove large, settleable, clay particles, and then used to attain the desired turbidity in the test water prior to each experiment. Stock solutions of coagulants were prepared using deionized water.

For the filtration experiments, a column was constructed from an acrylic plastic cylinder. It was 1.83 m in length and had an inside diameter of 3.81 cm. The filter media consisted of washed sand having a D_{60} of 0.53 mm and a D_{10} of 0.38 mm, with a uniformity coefficient of 1.4. The depth of sand in the column was 0.91 m and was supported by well-ground gravel, graded into three sizes. The base support was a 16.5 cm layer of gravel with a diameter in excess of 4.7 mm. The middle layer was 3.8 cm of gravel with a diameter between 3.36 and 4.7 mm, while the upper layer of the underdrain was gravel having a diameter between 2.36 and 3.36 cm and was 2.54 cm deep.

The sand column was backwashed immediately prior to each experiment. Dechlorinated tap water was used as the suspending medium. As will be noted later, one percent raw municipal wastewater was used as the organism inoculum in several experiments. In all other experiments, washed cell suspensions of *E. coli*, *C. parapsilosis*, *M. fortuitum*, and *M. phlei*, prepared according to the aforementioned procedures, were used as the source of organisms and were added at the same time to the influent to the filter. The inoculated water, having either the pure cultures or wastewater as the source of organisms, was prepared in a large volume and was continuously mixed and, at the same time, pumped to the head of the filter to maintain a standing head of between

15 and 35 cm of water. At periodic intervals during the course of each experiment, samples from the influent tank and from the filtrate were taken for microbial analysis.

Continuous Flow Ozonation Experiments

To study the relative effects of ozone bubbles and ozone residual on the inactivation of yeasts and acid-fast organisms, three different continuous flow reactor arrangements were devised. In arrangement No. 1, ozone demand free water, inoculated with the test organisms, was fed into the top of the reactor and removed from the bottom. A gaseous mixture of ozone and air was supplied at the bottom of the reactor through a fritted glass diffuser and exhausted through the top. The reactor consisted of a 55 mm Pyrex column 270 mm long, with a 500 mℓ volume. This arrangement provided information on the combined effects of ozone residual and ozone bubbles on the inactivation of the test organisms.

In arrangement No. 2, using the same physical reactor as above, inoculated water and sodium thiosulfate solution were fed into the top of the reactor through separate streams. The ozone/air mixture was supplied at the bottom of the reactor and exhausted through the top. The use of sodium thiosulfate reduced all residual dissolved ozone, permitting study of only the effect of ozone bubbles on organism inactivation. In arrangement No. 3, an aqueous solution of ozone was added at the top of the reactor along with a stream of organism-inoculated water. The reactor used in this arrangement was a Pyrex glass bottle having a diameter of 80 mm, a height of 120 mm, and a volume of 500 mℓ. A Teflon-coated magnetic stirring bar, driven by an external magnet, was used for mixing. In this arrangement the effect of ozone bubbles was eliminated, thus permitting study of the effect of ozone residual on organism inactivation.

A preliminary study using tracers confirmed that all three of the reactor arrangements approximated an ideal continuously-stirred tank reactor (CSTR). The three experimental arrangements are shown schematically in Figure 9.

The ozone demand free water used in these experiments was prepared by ozonating deionized water for 15 min, followed by boiling. To further ensure dissipation of any ozone residual, the water was exposed overnight to ultraviolet light.

The air/ozone mixture used in these studies was produced in a Welsbach T-408 Laboratory Ozonator (Welsbach, Philadelphia, PA) under a feed pressure of 0.703 kg/cm^2 gauge. The ozonator was operated at a pressure of 0.562 kg/cm^2 gauge and a voltage setting of 80-115 v. Feed air was supplied from the laboratory service line, dried with calcium chloride, and filtered through a 30 cm column of activated silica gel and fiberglass wool.

Initially, two dissolved ozone analyzers were employed in this study. However, due to poor instrument performance, analysis of dissolved ozone was performed using the method of Shechter (8). All experiments were performed

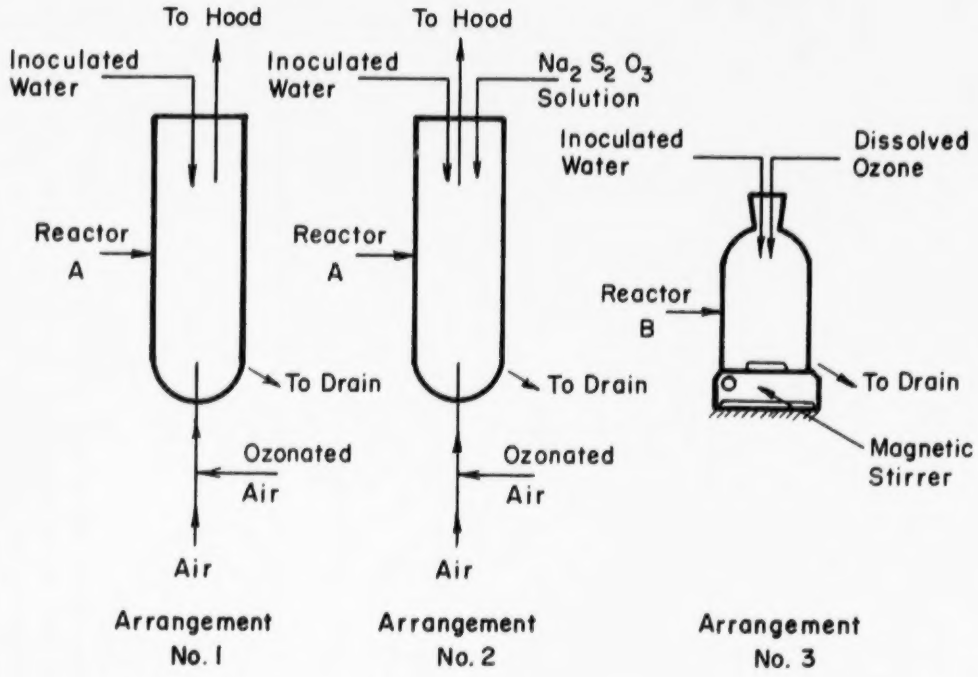
To Hood
Inoculated Water
Reactor A
To Drain
Ozonated Air
Air
Arrangement No. 1
To Hood
Inoculated Water
$Na_2S_2O_3$ Solution
Reactor A
To Drain
Ozonated Air
Air
Arrangement No. 2
Inoculated Water
Dissolved Ozone
Reactor B
To Drain
Magnetic Stirrer
Arrangement No. 3

Figure 9. Schematic Diagram of Reactor Arrangement Nos. 1, 2 and 3 Used in Ozone Inactivation Studies

using deionized water buffered at pH 7. The temperature of the water was maintained at 24°C throughout all experiments. The entire experimental setup was sterilized before use by passing a stream of ozonated air or ozonated water through the system for 15 min.

Mechanism of Inactivation by Chlorine

Hypochlorous acid used in these experiments was prepared according to the method of Moeller (10), yielding material low in chlorides. Radioactive hypochlorite was prepared by an isotope exchange between hydrochloric acid (^{36}Cl) and unlabeled hypochlorous acid (11,12).

Radioactivity analysis was performed in a Beckman LS-100 Liquid Scintillation Spectrometer (Beckman Instruments, Inc., Palo Alto, CA) using a 2:1 v/v mixture of phase-combining scintillant (Amersham Searle, Arlington Heights, IL) and scintillation grade p-xylene (Amersham Searle). Soluble radioactivity was measured by pipetting the sample directly into the scintillation cocktail; particulate radioactivity was measured by removing the particulate matter by either glass fiber (Schleicher and Schuell #25) or membrane filters (Millipore Type HAWP) and then placing them into the cocktail. Corrections were made, where necessary, for background and non-specific binding of radiotracer, but not for quench.

In the chlorine binding experiments, known aliquots of radio-labelled hypochlorite were added to erlenmeyer flasks containing CDFB. At appropriate intervals, the flasks were manually agitated and a sample withdrawn for particulate and total radioactivity analysis. Corrections were made for the binding of radio-labelled chloride. In determining the adsorption isotherms, equilibration times used were 30 min for C. *parapsilosis* and M. *fortuitum*, and 6 min for E. *coli*. The use of cell and chlorine controls was identical to that used in earlier studies (5,13).

The ability of microorganisms to grow after exposure to chlorine was assessed using a chlorine-exposed suspension of log phase cells and, after dechlorination with thiosulfate, resuspending the cells in fresh concentrated growth medium. The cells were incubated at the appropriate temperature, and growth was assessed by optical density measurements at periodic intervals.

Release of either ultraviolet-absorbing material or TOC by cells after chlorination was determined by exposing the test organisms to chlorine and dechlorinating aliquots withdrawn after periodic intervals. Cells were removed by centrifugation, and the supernate analyzed. The UV-absorbing material released was measured at 260 and 280 nm with a Beckman Acta III recording spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA), using quartz cuvettes of 1 cm pathlength in double beam mode referenced against a solution containing buffer and identical concentrations of thiosulfate and chlorine.

The effect of chlorine on cellular respiration was assessed by equilibrating cells with an appropriate substrate, which was found to have no chlorine demand. After a stable respiration rate was reached, as measured by a DO electrode, an aliquot of chlorine was added, and the resulting respiration rate was monitored. After 10 min of contact, thiosulfate was added and the final respiration rate assessed. Throughout each experiment, stirring was achieved by magnetic bar so that the incubation vessel could be closed to the atmosphere.

The effect of chlorine on cellular potassium uptake was determined using radiotracers. ^{42}K was prepared by irradiation of K_2CO_3 in the University of Illinois' TRIGA nuclear reactor. The cell suspension plus 1.5 g/ℓ dextrose was equilibrated for 5 min, after which ^{42}K was added. At appropriate intervals, samples were withdrawn for particulate radioactivity determination. At 45 min, chlorine was added and, after various time intervals, samples were withdrawn for radioactivity determination.

The effect of chlorination on protein synthesis, and DNA synthesis was determined using radiotracers. Log phase cells grown in the presence of 5 g/ℓ lactose were prepared. Chlorine demand free preparations of organisms were exposed to chlorine for 10 min in the case of *E. coli*, and 30 min for *C. parapsilosis* and *M. fortuitum*. After exposure and dechlorination, cells were split into two portions. To one portion of cells, 0.8 μCi/mℓ of (2,3,4,5-^{3}H)-L-Proline (Amersham Searle, Arlington Heights, IL) was added. At periodic intervals, samples were withdrawn and added to 1 volume of 10 percent (w/v) hot (70°C) trichloro-acetic acid (TCA). After contact at 70°C for 30 to 45 min, the treated cells were analyzed for particulate radioactivity subsequent to washing with cold TCA; radioactivity was interpreted as protein synthesis. To the second portion, 1.2 μCI/mℓ of (methyl-^{3}H)-thymine was added. At periodic intervals, samples were withdrawn and each was added to an equal volume of cold (5°C) TCA. After contact for 30 to 45 min, the treated cells were analyzed for particulate radioactivity after washing with cold TCA; radioactivity was interpreted as DNA synthesis.

SECTION 5

RESULTS AND DISCUSSION

ST. JOSEPH/OAKWOOD FIELD STUDIES

This investigation was carried out to ascertain the presence of the proposed indicator organisms in a typical wastewater treatment effluent, in the receiving stream above and below the outfall, and at a water treatment plant downstream. A related objective was to determine whether the proposed indicator organisms were ever absent in the presence of either fecal or total coliforms in such waters. It was also proposed to evaluate the diurnal and seasonal variability of these indicator organisms as related to the wastewater treatment plant, and the seasonal variability both in the receiving stream and at the downstream water treatment plant. The St. Joseph Wastewater Treatment Plant and its receiving stream, the Salt Fork of the Vermillion River, and the Oakwood Water Treatment Plant made up the system of study.

Analysis of yeast, acid-fast, and total and fecal coliform organisms was performed at the St. Joseph Wastewater Treatment Plant on samples of raw wastewater (SJ1), secondary effluent (SJ2), and the chlorinated effluent (SJ3) over a period of 24 hr on July 22-23, 1975 (Figure 2). Graphical variations of organism density as a function of time are presented in Figures 10 to 13. The results indicate the consistent occurrence of these organisms in the wastewater treatment plant. The density of yeast and acid-fast organisms in the raw wastewater was found to be approximately $10^4/100\ m\ell$. The density of fecal coliforms was consistently higher than the proposed indicator organisms by an order of 2-2.5 logs, while total coliforms were usually higher than fecal coliforms by a magnitude of one log.

The mean density of the four groups of organisms over the 24 hr period at the three sampling stations is presented in Figure 14. In general, a constant removal pattern of 1-1.5 log reduction for all four organism groups was observed through secondary treatment, contact stabilization process of aeration, and settling. It may be concluded that the four groups of organisms showed similar behavior with respect to the contact stabilization process. Reduction of organism density as a result of chlorination was observed to be in the range of 1-3.5 logs. The relative resistance of these four groups of organisms to chlorination may be determined from Figure 14 by comparing the slopes of the plot between points SJ2 and SJ3. It can be seen from this comparison that yeasts and total coliforms showed similar resistance, while acid-fast organisms were most resistant and fecal coliforms were

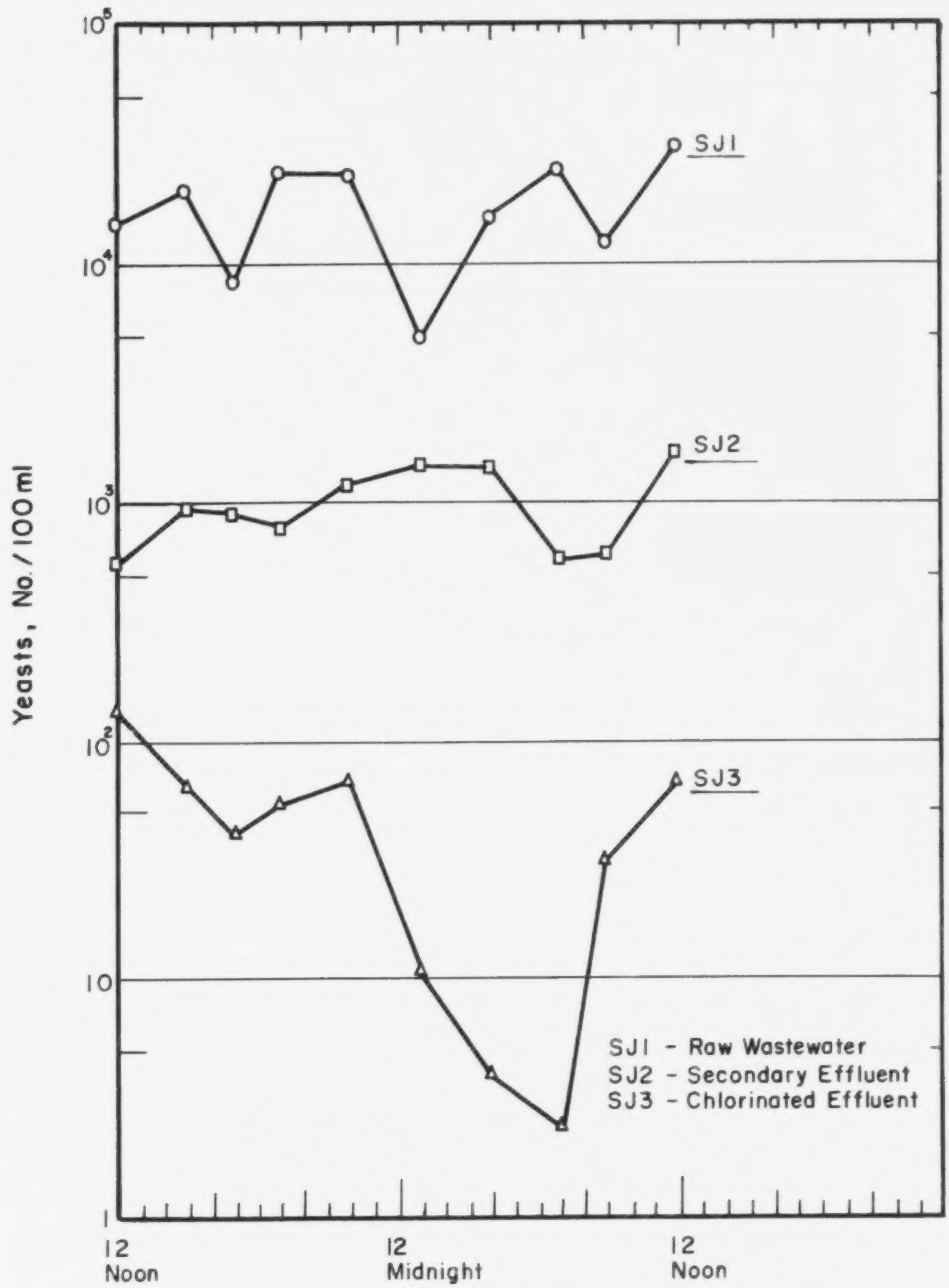

Figure 10. Densities of Yeasts at the St. Joseph Wastewater Treatment Plant Over a 24 Hr Period

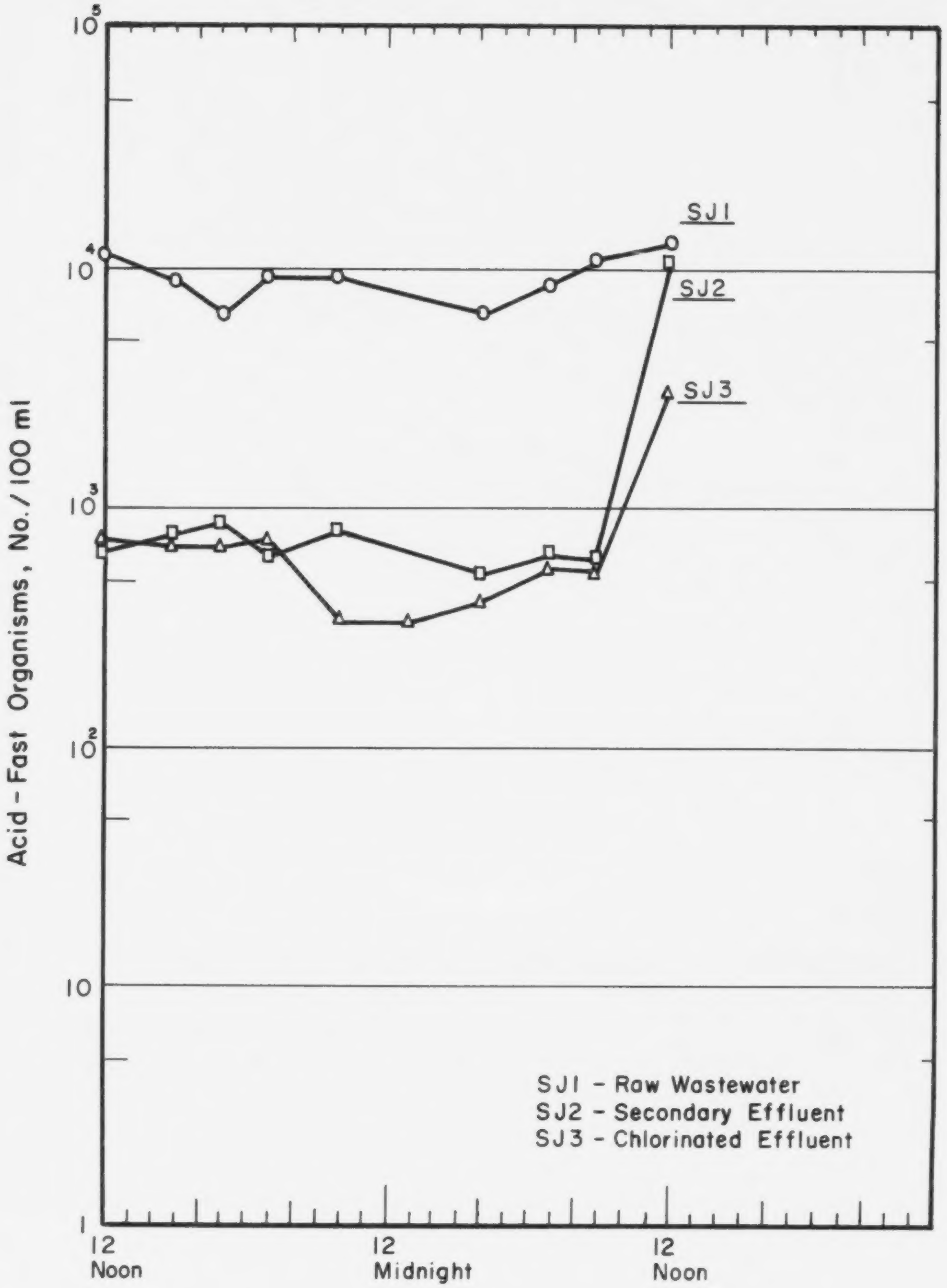

Figure 11. Densities of Acid-Fast Organisms at the St. Joseph Wastewater Treatment Plant Over a 24 Hr Period

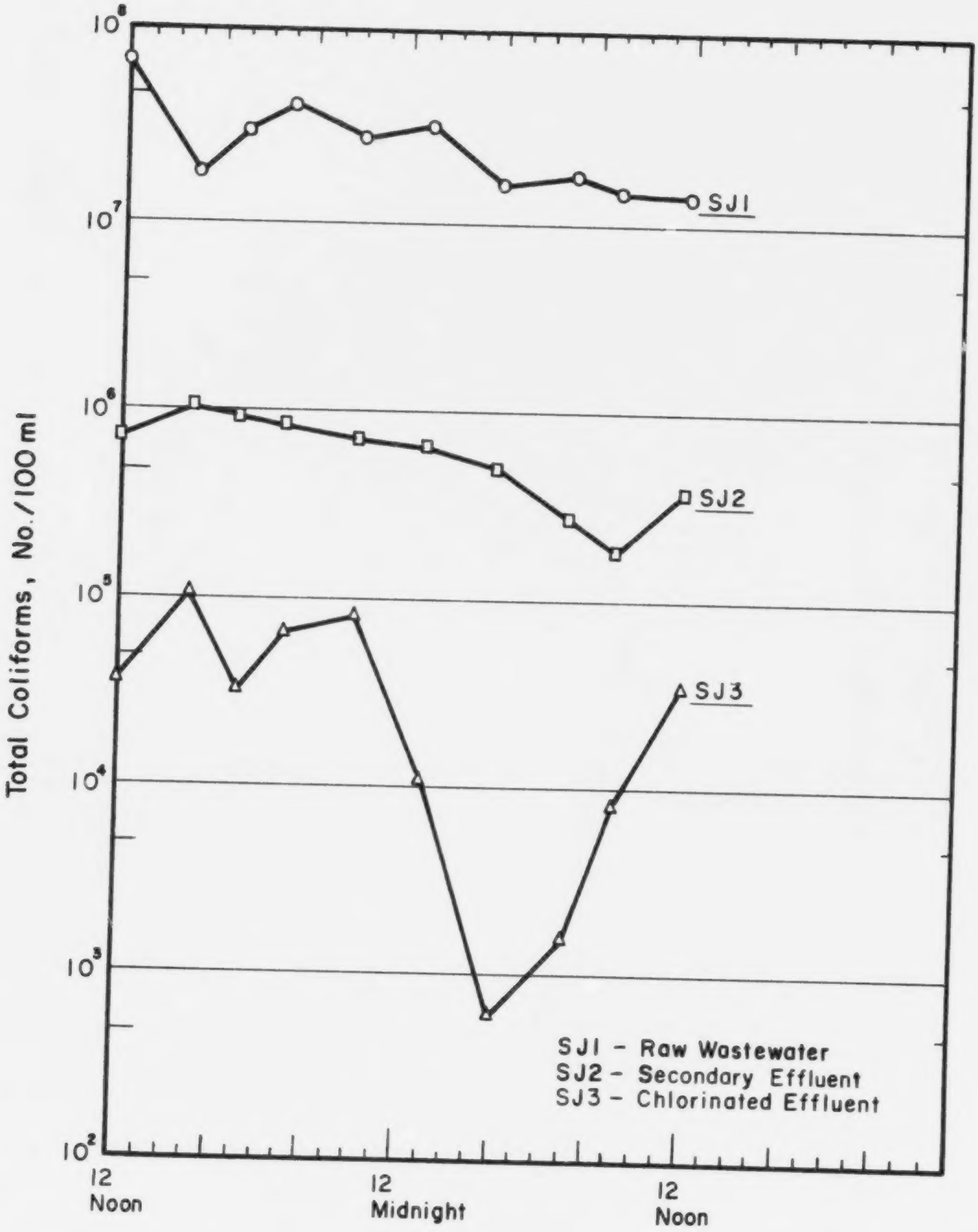

Figure 12. Densities of Total Coliforms at the St. Joseph Wastewater Treatment Plant Over a 24 Hr Period

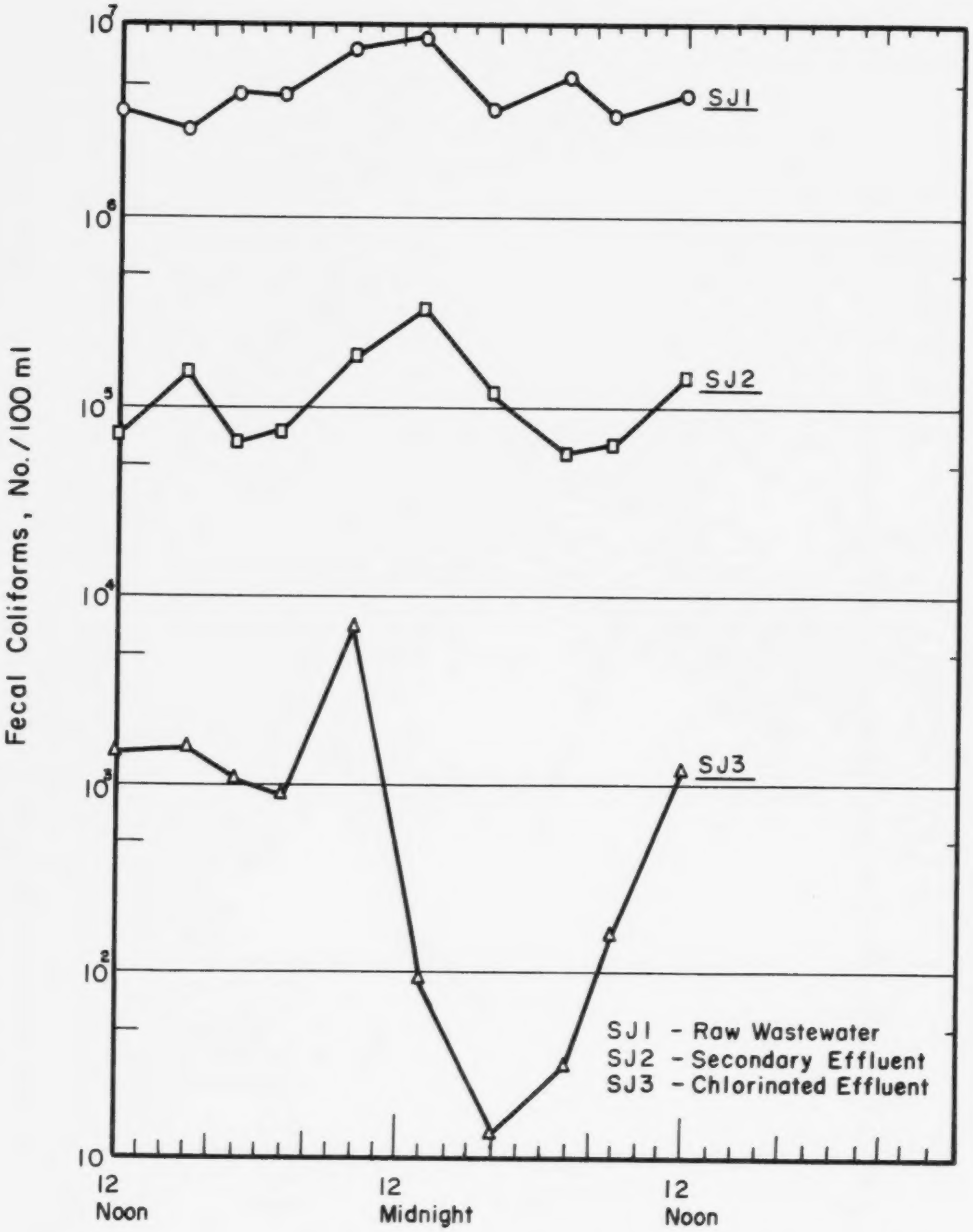

Figure 13. Densities of Fecal Coliforms at the St. Joseph Wastewater Treatment Plant over a 24 Hr Period

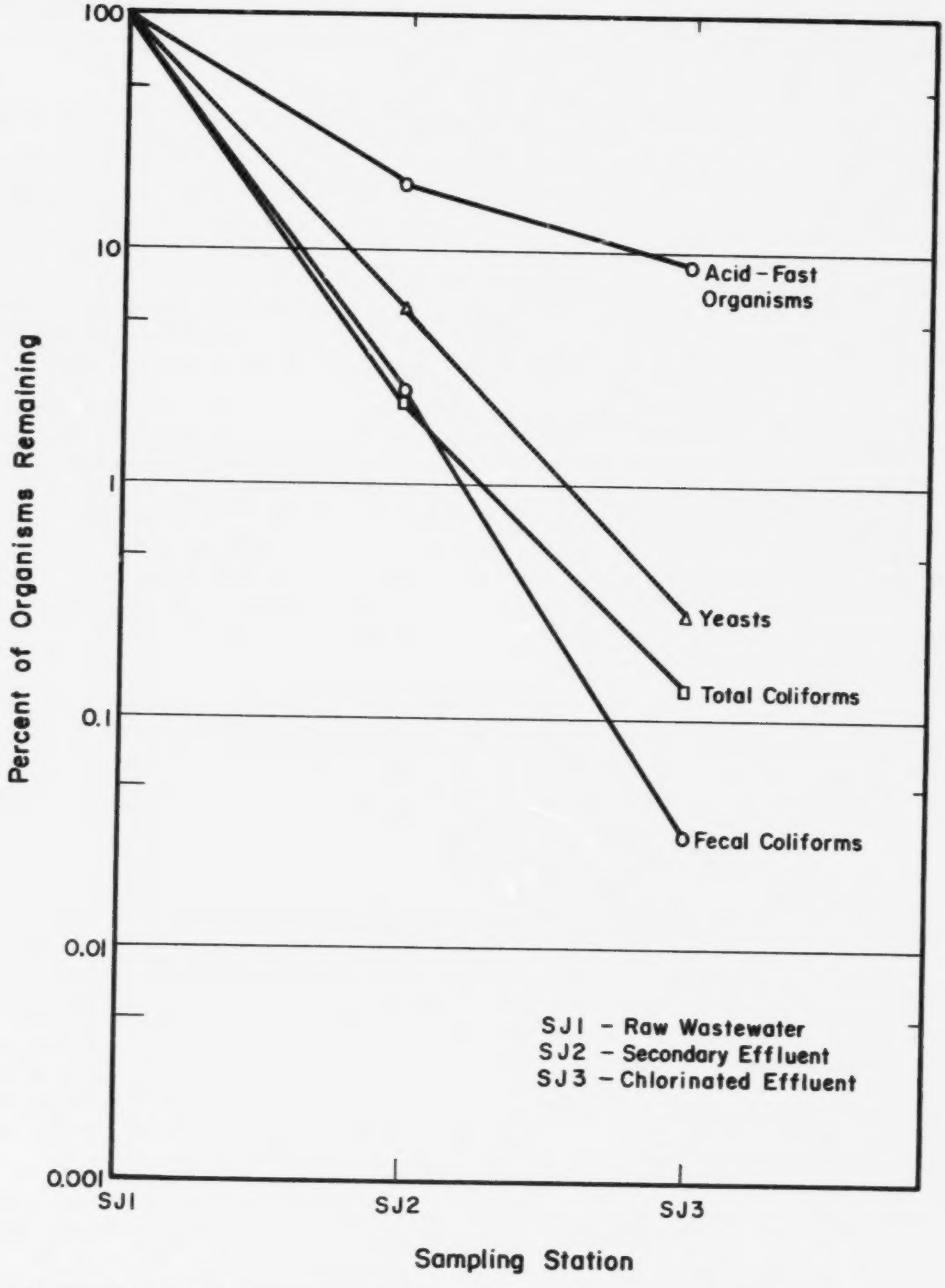

Figure 14. Mean Normalized Organism Densities at the St. Joseph Wastewater Treatment Plant Over a 24 Hr Period

the least resistant to disinfection by chlorine. A typical chlorine dosage ranged from 3.5-4.0 mg/ℓ and the plant effluent contained an average total chlorine residual of 0.8 mg/ℓ.

Seasonal variation of yeast, acid-fast, fecal, and total coliform organism density at the St. Joseph Wastewater Treatment Plant was evaluated by sampling the wastewater at the three sampling stations previously described during the period 2 May 1975 to 29 January 1976. The results are presented as three dimensional bar diagrams in Figures 15 to 18. The density of yeast, acid-fast, and fecal coliform organisms in the raw wastewater was generally low during the winter months (November to January) as compared with their density during warmer periods (May to October). Reductions in organism density through the contact stabilization process and as a result of chlorination were very similar to the results of the 24 hr study discussed above.

The density of yeasts in the raw wastewater at the St. Joseph Wastewater Treatment Plant varied from 2.73×10^3-3.94×10^4/100 mℓ during the period of study. A similar variation in the yeast density, 4.86×10^3-3.13×10^4/100 mℓ, was observed during the 24 hr study. Acid-fast organism densities during the same period of study varied from 1.43×10^3-1.43×10^4/100 mℓ in the raw wastewater. Less variation in the acid-fast density was observed in the raw wastewater during the 24 hr study, where it ranged from 6.50×10^3-1.26×10^4/100 mℓ. It is significant to note that yeasts and acid-fast organisms were always detected in the raw wastewater over the entire period of study.

The density of fecal coliforms in the raw wastewater varied from 6.67×10^4-9.45×10^6/100 mℓ during the period of study. This represents a two-log variation as compared with a one-log variation in the raw wastewater density of both yeast and acid-fast organisms. The variation in fecal coliform density during the 24 hr study was much less than that observed in the seasonal study, ranging from 2.96×10^6-9.26×10^6/100 mℓ. Enumeration of total coliforms in the raw wastewater was difficult due to their extreme variation in density. In almost all cases, the density of total coliforms was 0.5-2 logs greater than the fecal coliform density.

The Salt Fork River system was also examined to ascertain whether yeasts and acid-fast organisms were present whenever the commonly accepted indicator organisms (fecal and total coliforms) were present in such waters. Data were also collected and analyzed to establish the variability, both temporal and spatial, of yeasts and acid-fast organisms in the river system and to compare their variability to that of the coliform group of organisms. The location and description of the sampling stations for the Salt Fork River system are given in Figure 4 and Table 6.

The density of yeasts, acid-fast organisms, fecal coliforms, and total coliforms in the Salt Fork River system are presented in Figures 19 to 22. It should be noted that the chlorinated wastewater effluent from the St. Joseph Wastewater Treatment Plant is introduced into the river system between stations RS0 and RS1. Although somewhat difficult to determine from Figure 21, the density of fecal coliforms decreased continuously between RS1 and RS5

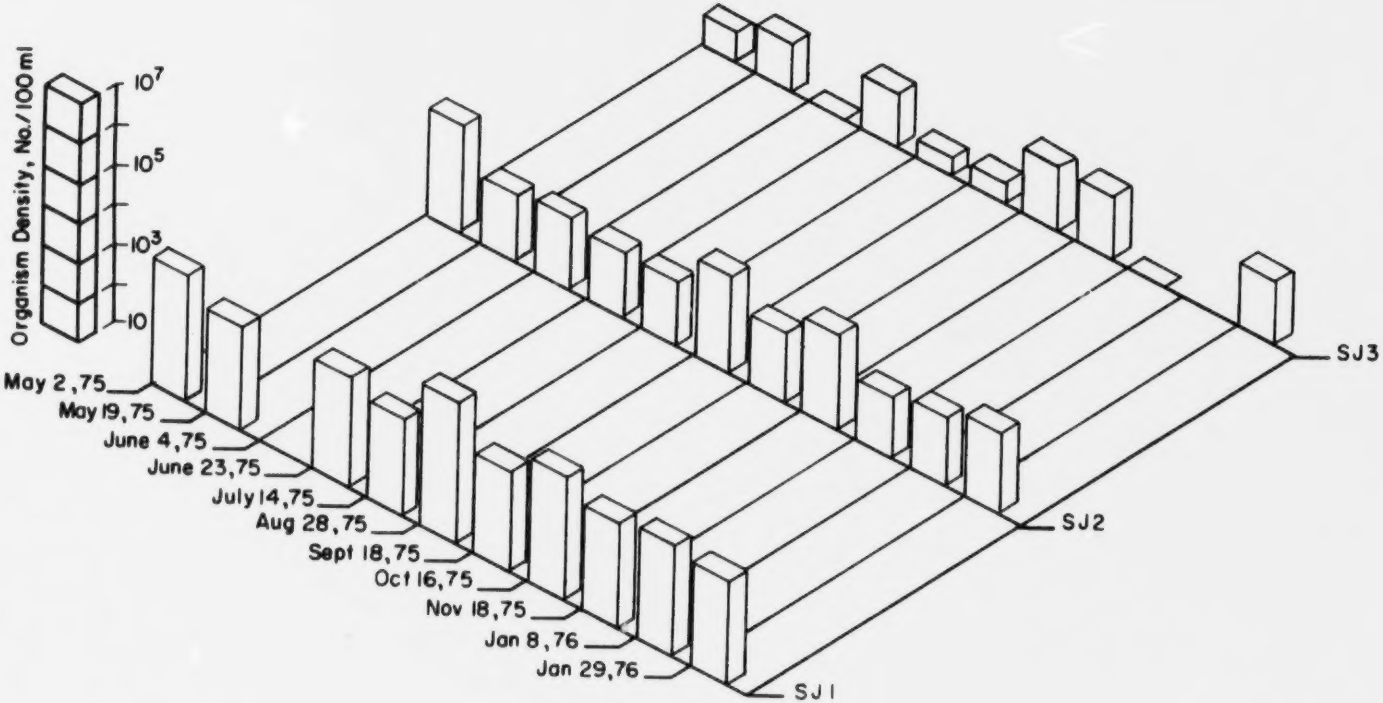

Figure 15. Densities of Yeasts at the St. Joseph Wastewater Treatment Plant

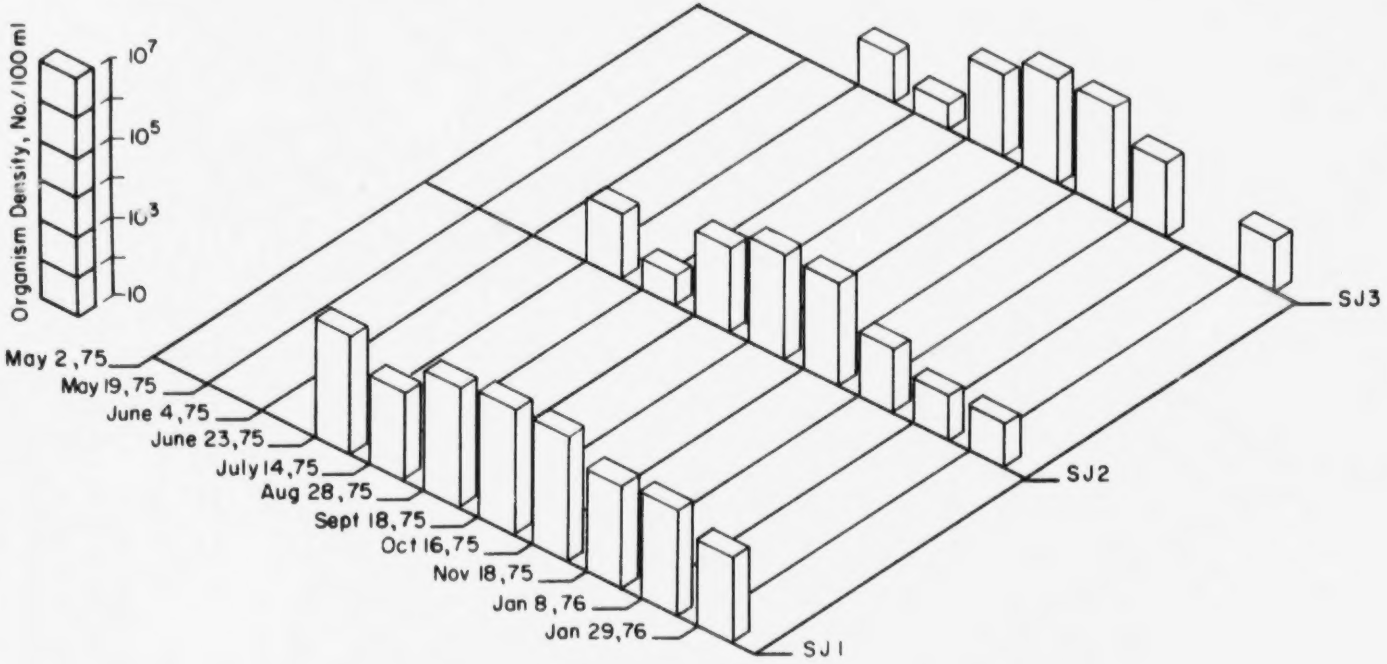

Figure 16. Densities of Acid-Fast Organisms at the St. Joseph Wastewater Treatment Plant

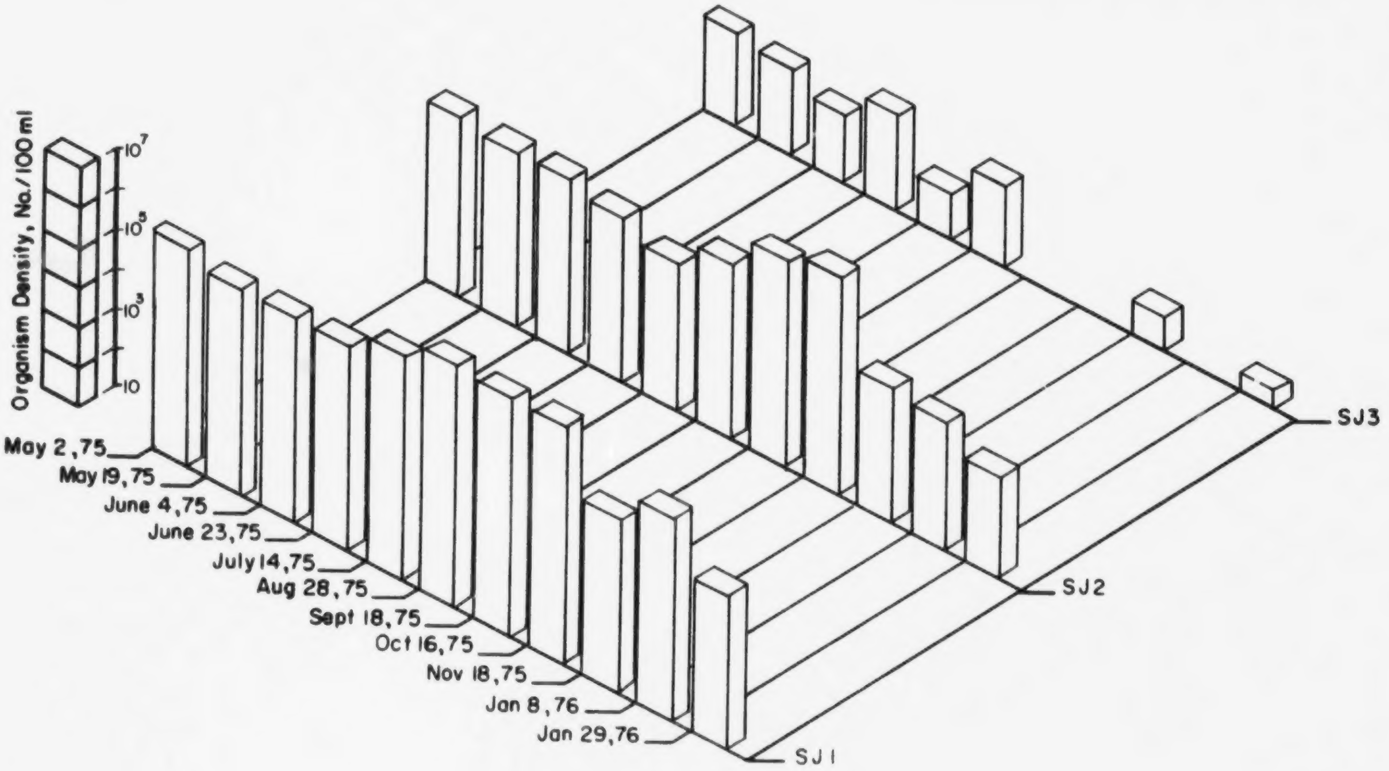

Figure 17. Densities of Fecal Coliforms at the St. Joseph Wastewater Treatment Plant

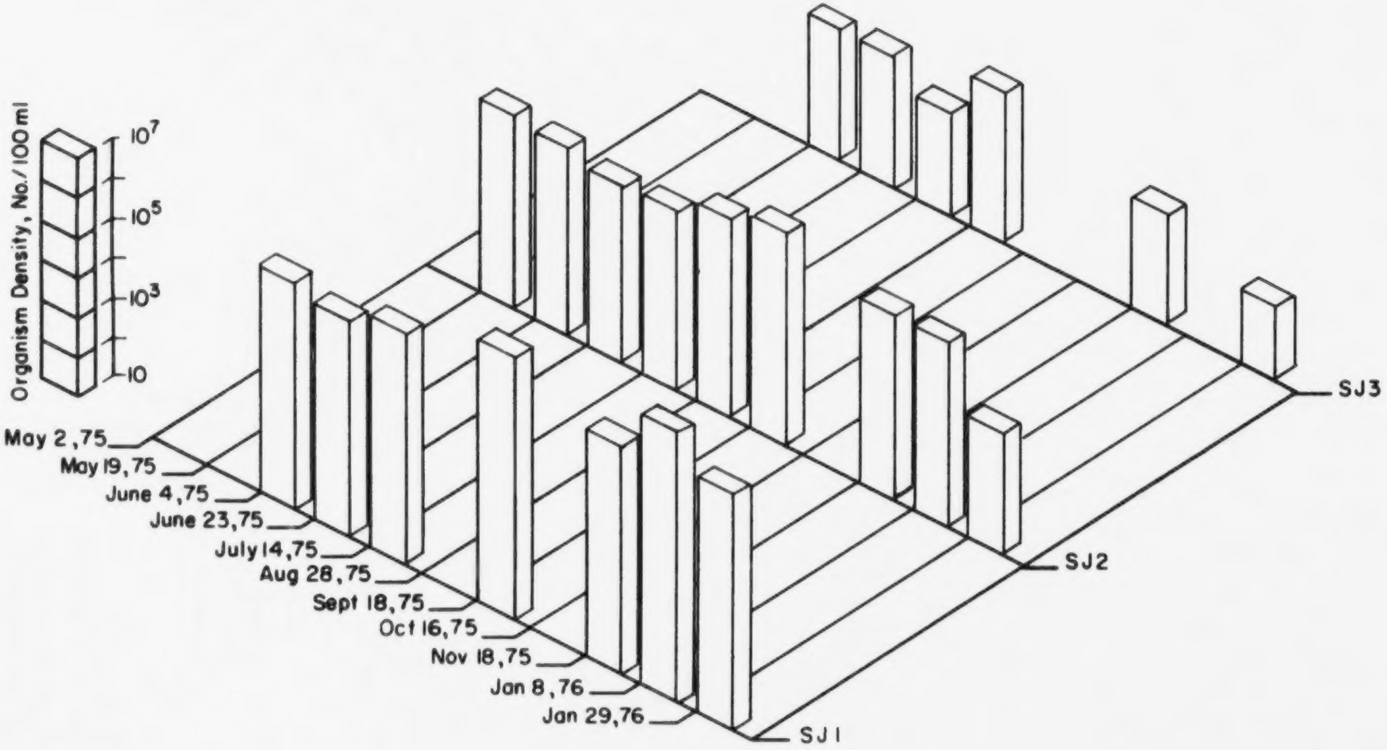

Figure 18. Densities of Total Coliforms at the St. Joseph Wastewater Treatment Plant

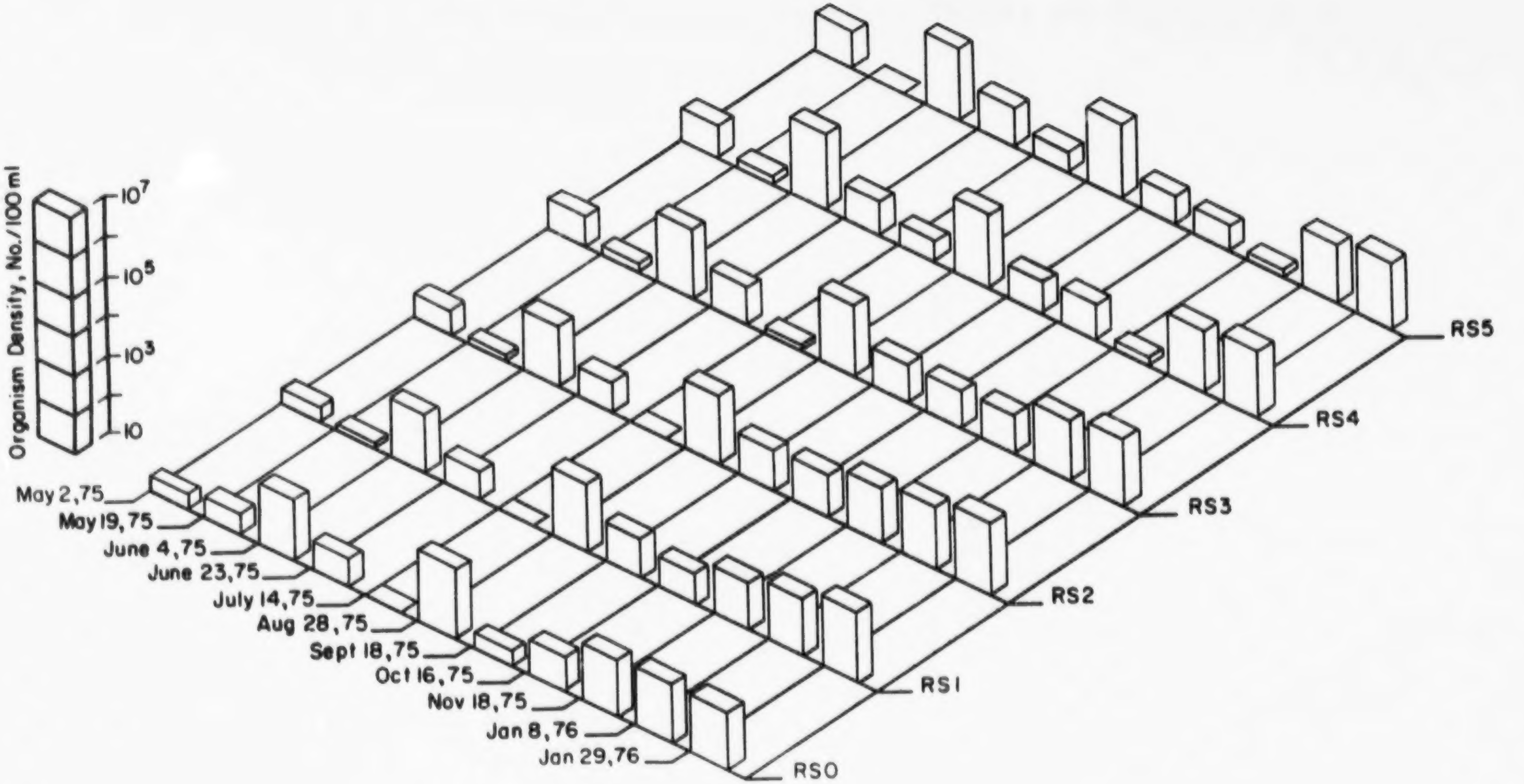

Figure 19. Densities of Yeasts in the Salt Fork River System

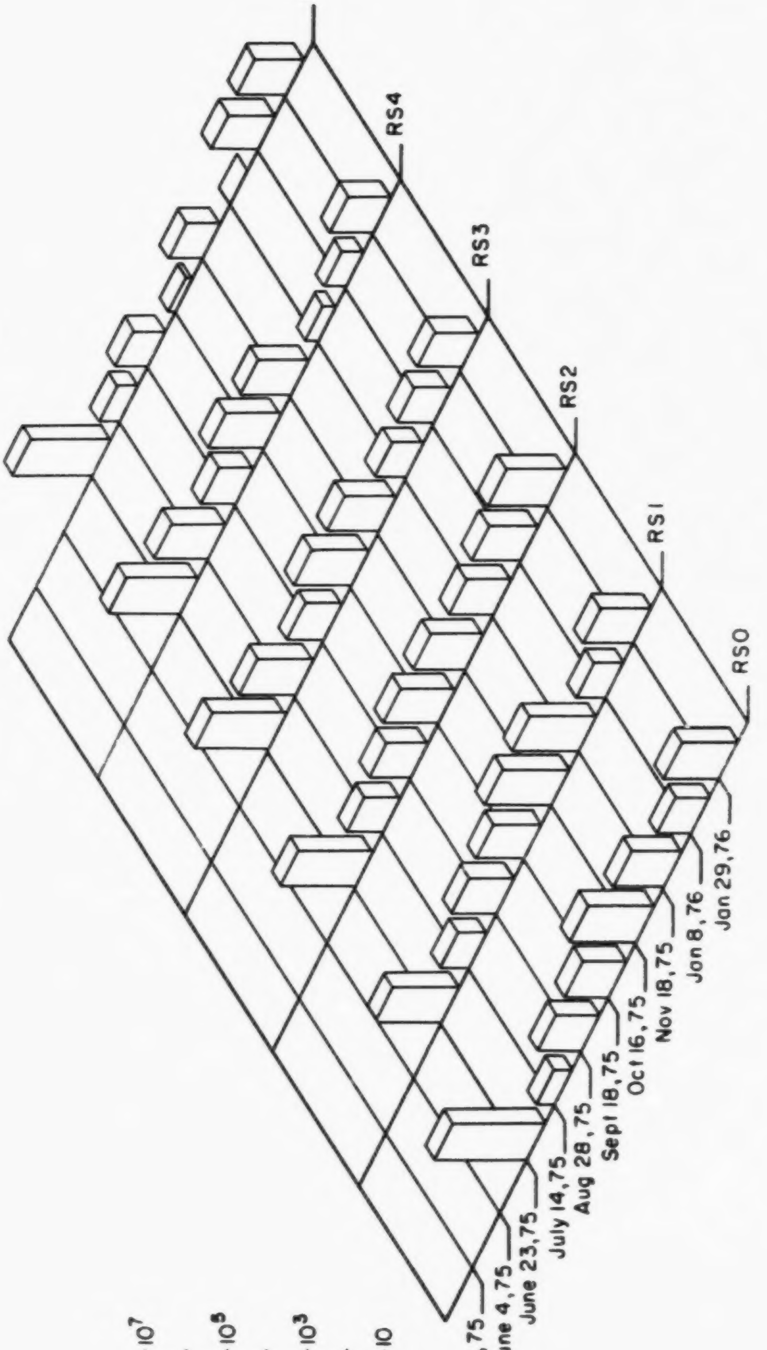

Figure 20. Densities of Acid-Fast Organisms in the Salt Fork River System

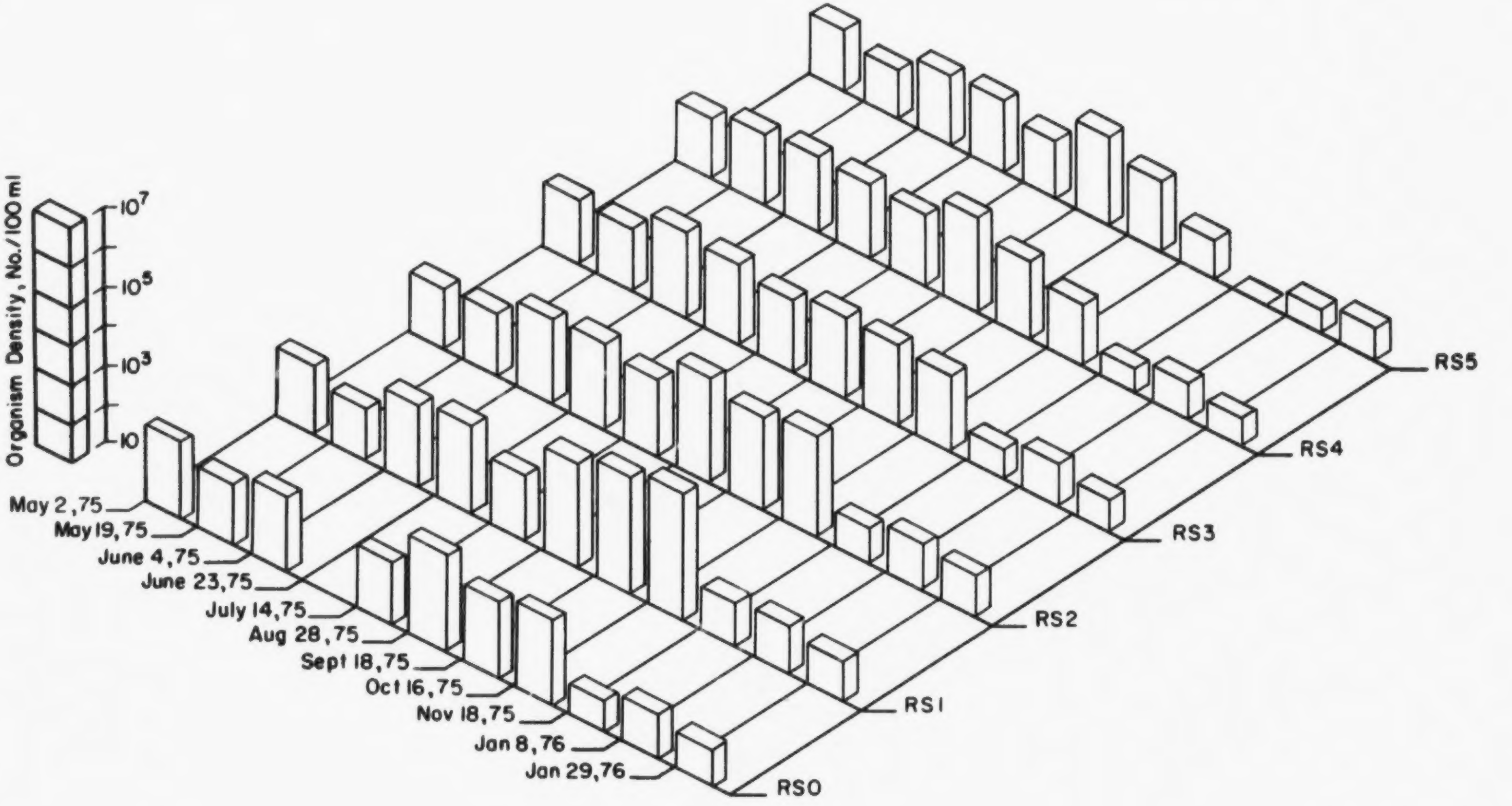

Figure 21. Densities of Fecal Coliforms in the Salt Fork River System

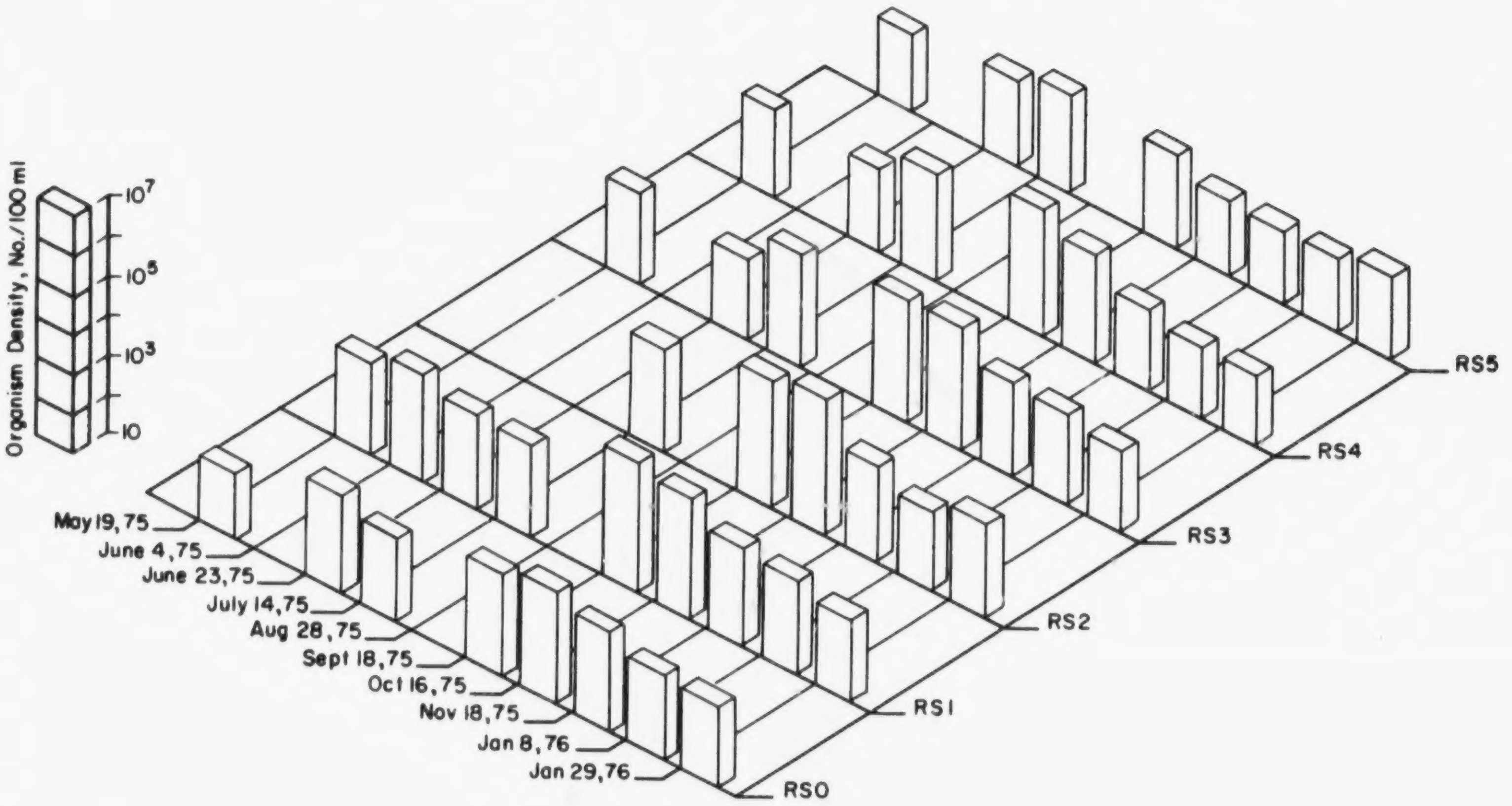

Figure 22. Densities of Total Coliforms in the Salt Fork River System

in the field samples collected on June 23, September 18, October 16, and November 18, 1975, and in those on January 8 and 29, 1976. A maximum decrease in fecal coliform density of 2.5 logs was observed from RS1 to RS5 based upon the results of samples obtained on October 16. The samples collected on the other dates did not show a definite trend with respect to a change in density of fecal coliforms. Figure 19 indicates a continuous increase in yeast density on two occasions between RS1 and RS5 (May 2, 1975 and July 14, 1975). A continuous decrease in yeast density was observed on September 18 and November 18, 1975. The density of yeasts in the river system remained relatively constant between RS1 and RS5 on the other sampling dates. The density of acid-fast organisms in the river system fluctuated from station to station on most of the sampling dates (Figure 20). Exceptions to this observation occurred on October 16, 1975, when there was a minor decrease in density, and on August 23, 1975 and November 18, 1975, when there was a continuously decreasing trend in the density of acid-fast organisms. The enumeration of total coliform organisms was difficult in the river samples due to their extreme variability (Figure 22). It was observed, however, that the density of total coliforms was less during the cold periods than during the warmer months.

Differences in the density of each of the four groups of organisms at sampling stations located above and below the point of discharge of chlorinated secondary effluent by the St. Joseph Wastewater Treatment Plant were not found to be significant, i.e., the wastewater effluent did not appear to cause significant changes in the biological quality of the Salt Fork River water in terms of the organism groups studied. Physical-chemical parameters monitored above and below the outfall of the wastewater treatment plant also showed that the effluent had limited impact on the quality of water in the stream.

The density of the four organism groups was also determined at four stations within the Oakwood Water Treatment Plant system (Figure 3). The results are presented in Table 9. During the sampling period covered by this investigation, the density of all four groups of organisms in the raw water reservoir (OW1) was much lower than their respective density in the Salt Fork River system. This may have been due to the settling and dieoff of the organisms in the holding reservoir; the addition of copper sulfate to control algal growth in the reservoir during the summer months may also have had an effect. The majority of the organisms present at OW1 were removed from the water by the process of coagulation-flocculation, prechlorination, and filtration. In most of the samples analyzed, none of the test organisms could be detected in the filtered water (OW3), and in almost all cases, any organisms present at OW3 were inactivated by chlorine during the disinfection process, resulting in their absence in the finished water at station OW4.

DECATUR FIELD STUDIES

A field sampling program was performed at Decatur, Illinois, over a seven-month period to analyze the occurrence of the proposed indicator organisms through a water treatment plant and in its associated distribution

TABLE 9. DENSITY OF ORGANISMS AT THE OAKWOOD WATER TREATMENT PLANT

Sampling Date	Yeasts no./100 mℓ				Acid-fast organisms no./100 mℓ				Fecal coliforms no./100 mℓ				Total coliforms no./100 mℓ			
	OW1	OW2	OW3	OW4	OW1	OW2	OW3	OW4	OW1	OW2	OW3	OW4	OW1	OW2	OW3	OW4
3/4/75	0			0						0		0				
4/4/75	9.00			0					5.50			0				
5/2/75	0.33	0	0	0					0	0	0	0				
5/19/75	0	0	0	0					0	0	0	0			0	0
6/4/75	125.00	0.33	0	0					3.30	0	0	0		0	0	0
6/23/75	5.50	0.67	0.34	0	10.00	0	0	0	0	0	0	0	0	0	0	0
7/14/75	9.00	0	0	0	65.00	53.00	0.67	0	0.50	0	0	0	29.68	0.51	0.26	0
8/28/75	0.67	0	0	0	6.67	0	0	0	0	0	0	0		0	0	0
9/18/75	14.00	0	0	0	12.00	7.67	0	0		0	0	0		0	0	0
10/16/75	3.50	0	0	0	20.00					0.67	0	0.67		7.34	1.50	1.00
11/18/75	0.34	0	0	0	13.34	0	1.00	0.67	0.34	0	0	0	1.67	0	0	0
1/8/76	78.34	22.70	0.67	0	110.00	3.00	2.30	0	8.67	2.34	0	0		8.75	0.34	0
1/29/76	106.00	1.00	0	0	17.00	3.67	0.34	0	0.34	0	0	0	11.34	0	0	0

system. Previously described enumeration techniques were used to analyze large-volume samples for yeasts, acid-fast organisms, total coliforms, and standard plate count bacteria. A complete description of the treatment plant, distribution system, and sample stations has been given (Figure 5 and Table 8).

Total coliform analysis by MF and MPN techniques revealed that coliform organisms were always present in the influent raw water (station No. 1). The density of total coliform organisms in the influent raw water as determined by the MF technique ranged from 190-29,800/100 mℓ over the entire sampling period (Table 10). Total coliform densities as determined by the MPN technique were less than by MF in all cases, with a range of 31- $\geq$2400/100 mℓ. The mean log difference between the MF and MPN techniques in determining total coliforms in the raw water was 0.825, i.e., MF > MPN. Table 10 shows that coliforms were found in the treated water on two occasions, at station No. 2 on 23 August 1977, and at station Nos. 4 and 5 on 11 October 1977. The presence of coliforms at two of these three sample stations was confirmed by the MPN technique (Table 11). The occurrence of coliforms at station No. 4 on 11 October 1977 was not confirmed by the MPN technique.

TABLE 10. DENSITY OF TOTAL COLIFORM ORGANISMS DURING DECATUR FIELD SAMPLING (No./100 mℓ by the MF Enumeration Technique)

Date	Sampling Station				
	1	2	3	4	5
4/05/77	190	0	0	0	0
4/19/77	290	0	0	0	0
5/24/77	1680	0	0	0	0
6/01/77	1480	0	0	0	0
6/07/77	1200	0	0	0	0
6/14/77	190	0	0	0	0
6/28/77	464	0	-	0	0
7/06/77	4900	0	0	0	0
7/19/77	673	0	0	0	0
8/23/77	1990	132	0	0	0
9/07/77	29800	0	0	0	0
9/20/77	7400	0	0	0	0
9/27/77	5800	0	0	0	0
10/11/77	5530	0	0	0.5	30

TABLE 11. DENSITY OF TOTAL COLIFORM ORGANISMS DURING DECATUR FIELD SAMPLING (No./100 mℓ by the MPN Enumeration Technique)

Date	Sampling Station				
	1	2	3	4	5
4/05/77	70	*	*	*	*
4/19/77	31	*	*	*	*
5/24/77	240	*	*	*	*
6/01/77	170	*	*	*	*
6/07/77	110	*	*	*	*
6/14/77	170	*	*	*	*
6/28/77	170	*	-	*	*
7/06/77	33	*	*	*	*
7/19/77	70	*	*	*	*
8/23/77	920	>16	*	*	*
9/07/77	≥2400	*	*	*	*
9/20/77	1600	*	*	*	*
9/27/77	350	*	*	*	*
10/11/77	≥2400	*	*	*	5.1

*Results expressed as organism density <2.2/100 mℓ

Yeasts were detected with 100 percent frequency in the influent raw water during the sampling period; the geometric mean value was 5.7/ℓ. Yeasts were enumerated in the finished tap water at the treatment plant (station No. 2) with 15 percent frequency; the geometric mean density was 5.3/ℓ. No yeasts were recovered from stations Nos. 3 and 4 in the distribution system. The most terminal sampling point in the distribution system (station No. 5) showed the occurrence of yeasts in two samples, or in 15 percent of the samples examined. These results are summarized in Table 12.

Acid-fast organisms were recovered from all of the influent raw water samples examined (Table 13). The frequency of recovery from a potable water tap at the treatment plant (station No. 2) was 36 percent, and the incidence of acid-fast organisms in the distribution system ranged from 7-23 percent. The density of acid-fast organisms in the influent raw water ranged from 6-169/ℓ, with a geometric mean of 37.0/ℓ. Table 13 summarizes the results of the occurrence of acid-fast organisms during this study; it should be noted that these organisms were detectable at all sampling stations at one time or another. Chlorination and other treatment processes caused a reduction in

TABLE 12. DENSITY OF YEAST ORGANISMS DURING DECATUR FIELD SAMPLING (No./ℓ)

Date	Sampling Station				
	1	2	3	4	5
4/05/77	16	0	0	0	0
4/19/77	20	0	0	0	0
5/24/77	6	0	0	0	0
6/01/77	5	0	0	0	0
6/07/77	3	0	0	0	0
6/14/77	7	0	0	0	0
6/28/77	9	0	-	0	0
7/06/77	3	0	0	0	0
7/19/77	3	0	0	0	0
8/23/77	12	9.5	0	0	0.5
9/07/77	1	0	0	0	1
9/20/77	2	3	0	0	0
9/27/77	16	0	0	0	0
10/11/77	-	-	-	-	-

the density of acid-fast organisms within the treatment plant of between 27 and 100 percent.

Bacterial densities as measured by the estimated standard plate count (SPC) were found to be very high. At sample station No. 1 (raw water influent), the SPC values ranged from 4850-48,840/mℓ, with a geometric mean density of 19,879/mℓ. Of particular interest was the fact that between 14 and 43 percent of the total number of samples obtained from the distribution system showed a SPC which exceeded that of the SPC in the raw water influent (station No. 1, Table 14). It should be noted that the SPC values at station No. 4 on 7 June 1977 and 19 July 1977 were on the order of 10^6 and 10^5/mℓ, respectively. These values appear to be unreasonably high relative to the other data for station No. 4 and, therefore, may not be an accurate indication of the existing bacterial quality in the distribution system at this sampling station. Alternatively, the high bacterial counts observed at station No. 4 on these dates might have been due to the dislodgement of biological slimes by high flow conditions in the main. As previously mentioned, the fact that the North Treatment Plant may contribute some flow at this location in the distribution network could also account for the observed high densities.

TABLE 13. DENSITY OF ACID-FAST ORGANISMS DURING DECATUR FIELD SAMPLING (No./ℓ)

Date	Sampling Station 1	2	3	4	5
4/05/77	61	0	0	0	0
4/19/77	6	0	0	0	0
5/24/77	108	0	0	0	0
6/01/77	51	0	0	0	0
6/07/77	33	24	1	1	2
6/14/77	48	4	2	3	0
6/28/77	100	0	-	0	0
7/06/77	8	0	0	0	0
7/19/77	93	0	0	0	0
8/23/77	96	5	2.5	6	0
9/07/77	48	1	0	0	0
9/20/77	6	0	0	0	0
9/27/77	8	0.5	0	0	0
10/11/77	169	0	0	0	0

It may be calculated that the treatment processes, including chlorination, lead to a 13-98 percent reduction in the SPC between sample stations Nos. 1 and 2 (Table 14). On 28 June 1977, however, the number of bacteria as determined by the SPC actually increased between the raw and the treated water. Table 15 gives the geometric mean density for each of the organism groups at each of the sample stations. The increase in SPC as seen at station No. 4 was due to the inclusion of the data from 7 June 1977 to 19 July 1977 as previously mentioned. If these two values are omitted due to their questionable accuracy, the geometric mean SPC at station No. 4 becomes 5729/mℓ.

A statistical analysis was performed on the organism density data to determine if any correlation might exist between the occurrence of any two organism groups in the influent raw water, particularly between coliforms and either the yeasts or acid-fast organisms. Product-moment correlation coefficients were also computed for raw water turbidity vs. log organism density and for raw water temperature vs. log organism density (14). Results of this analysis are given in Table 16. It can be seen that the only significant correlation among the parameters was an inverse correlation between yeast and total coliform organism density, at the 5 percent confidence level, i.e.,

TABLE 14. ESTIMATED STANDARD PLATE COUNT DURING DECATUR FIELD SAMPLING (No./mℓ)

Date	Sampling Station				
	1	2	3	4	5
4/05/77	15,940	290	490	3,050	9,270
4/19/77	19,080	6,490	18,270	910	4,440
5/24/77	20,135	2,000	23,870	4,310	28,960
6/01/77	4,850	2,280	1,040	7,440	8,935
6/07/77	34,260	6,290	6,200	$>10^6$	39,030
6/14/77	10,880	1,080	1,785	7,370	1,880
6/28/77	20,880	31,080	-	2,780	26,260
7/06/77	47,840	41,800	25,460	28,420	590
7/19/77	37,780	14,880	9,660	190,820	8,650
8/23/77	44,660	18,630	15,690	22,650	10,830
9/07/77	14,280	11,630	30,780	32,720	9,530
9/20/77	16,880	12,390	14,710	1,620	20,830
9/27/77	30,700	19,760	24,270	3,510	41,940
10/11/77	10,900	140	15	0	103

there was a negative association between yeast and total coliform density in the raw water samples.

Water leaving the treatment plant for distribution contained a free available chlorine residual ranging from 0.6-1.2 mg/ℓ, with a mean value of 0.88 mg/ℓ. In only 3 of the 14 series of samples was there no free chlorine residual detected at the most terminal sampling point of the distribution main under study (station No. 5).

TABLE 15. GEOMETRIC MEAN VALUES OF ORGANISM DENSITY AND CHLORINE RESIDUAL DURING DECATUR FIELD SAMPLING BETWEEN 4 APRIL 1977 and 11 OCTOBER 1977

Parameter	Sampling Station 1	2	3	4	5
Free Cl_2 residual, mg/ℓ	-	0.88	0.70	0.52	0.36
Total Cl_2 residual, mg/ℓ	-	1.09	1.06	0.82	0.40
Total Coliform					
MPN/100 mℓ	238.5 (14/14)*	**	**	**	**
MF/100 mℓ	1592.2 (14/14)	132 (1/14)	0.0 (0/13)	0.5 (1/14)	30 (1/14)
Yeast/ℓ	5.7 (13/13)	5.3 (2/13)	0.0 (0.12)	0.0 (0/13)	0.7 (2/13)
Acid-fast/ℓ	37.0 (14/14)	3.0 (5/14)	1.7 (3/13)	2.6 (3/14)	1.4 (1/14)
Est. Standard Plate Count/mℓ	19,879 (14/14)	5114 (14/14)	5096 (13/13)	11,160 (13/14)	7178 (14/14)

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[*] Numbers in parentheses indicate frequency of samples which were positive, e.g., 14/14, 14 positive out of 14 samples examined.

[**] MPN data for stations Nos. 2 through 5 were always <2.2/100 mℓ, except on two occasions:

23 August 1977 (station No. 2): count was >16/100 mℓ
11 October 1977 (station No. 5): count was 5.1/100 mℓ

TABLE 16. STATISTICAL CORRELATION ANALYSIS OF RAW WATER PARAMETERS FOR DECATUR FIELD STUDIES BETWEEN 4 APRIL 1977 and 11 OCTOBER 1977; NUMBER OF SAMPLES = 14

Variables	Correlation coefficient	Significance at the 5% level
TC and yeast densities	-0.593	Significant
TC and AF densities	-0.174	Not significant
Yeast and AF densities	-0.231	Not significant
Water Temp. and TC density	0.377	Not significant
Water temp. and AF density	-0.070	Not significant
Water temp. and yeast density	-0.544	Not significant
Water temp. and SPC density	0.132	Not significant
Turbidity and TC density	-0.198	Not significant
Turbidity and AF density	0.245	Not significant
Turbidity and yeast density	0.020	Not significant
Turbidity and SPC density	0.127	Not significant

AF = acid-fast organisms
TC = total coliform organisms
SPC = estimated standard plate count

CONTINUOUS FLOW CHLORINATION EXPERIMENTS

Continuous flow chlorine-inactivation experiments were performed to verify the resistance of the acid-fast, yeast, and coliform organisms under dynamic conditions and to investigate the inactivation behavior of the organisms under conditions more closely resembling that of water and wastewater chlorination. In an attempt to reproduce these conditions, a plug flow tubular reactor was used as the disinfection contact chamber and both pure culture and natural population inactivation studies were conducted. The pure culture chlorination studies were performed as a "mixed culture" of the test organisms in all cases; this permitted a direct comparison of the relative resistance of the organisms to chlorine under the same, simultaneous conditions. The natural population studies consisted of chlorinating a clarified activated sludge effluent and, since this effluent was also a "mixed culture" of the test organisms, all experiments established in a comparable way the relative resistance of the yeast, acid-fast, and coliform organisms to chlorine under constant experimental conditions.

Mixed Culture Studies

In the mixed pure culture studies the yeast, C. *parapsilosis*, the acid-fast, M. *fortuitum*, and the E. *coli* organisms were added together in CDFB and subjected to a free available residual chlorine concentration of between 0.515 and 1.620 mg/ℓ for contact times up to 20 min. The initial organism density used in these studies was approximately 7 x 10^5 mℓ for E. *coli*; the density ranged from 4.19 x 10^5-1.65 x 10^7/100 mℓ for M. *fortuitum*, and from 6.12 x 10^5-6.76 x 10^6/100 mℓ for C. *parapsilosis*. Experiments were performed at pH 7 and 10, and at room temperature which varied from 22°-24.5°C. Using these experimental conditions, it was possible to study the response of the test organisms to a range of free available chlorine residuals and ratios of hypochlorous acid to hypochlorite ion concentrations. It was also possible under these conditions to compare, to a certain degree, the results obtained in these experiments with those obtained in the batch inactivation studies.

The response of the "mixed culture" of organisms to free available residual chlorine is shown in Figures 23 and 24. The results shown in these two figures are typical in that only one concentration of free available chlorine is given for the two pH values studied; the response of the three organisms was also determined using other concentrations of chlorine at both pH 7 and 10 (Figures 25 through 28). In all the experiments, at least 4 logs of inactivation of E. *coli* occurred in the shortest contact time studied, 4 min. Because of the nature of the experimental reactor plus the high rate of inactivation, it was impossible to successfully enumerate E. *coli* during the first 4 min of contact. Acid-fast organisms were observed to have the highest degree of resistance to free residual chlorine, with yeast and coliform organisms showing progressively less resistance, respectively. In all experiments, the response of the mixed culture of yeast and acid-fast organisms to free residual chlorine clearly demonstrates the superior chlorine resistance of both of these groups of organisms over that of the coliforms. Figures 25 and 26 show the response of the yeast and acid-fast organisms, respectively, to various levels of free residual chlorine at pH 7 while Figures 27 and 28 summarize the response of the test organisms at pH 10.

At pH 7 and 20°C, approximately 80 percent of the free available residual chlorine is in the form of hypochlorous acid, whereas at pH 10, only approximately 0.4 percent is hypochlorous acid with the rest being hypochlorite ion. In general, hypochlorous acid is accepted as being a more potent disinfectant than hypochlorite ion in the inactivation of microorganisms. The results of this study support this observation in that it was shown that as the pH of the system increased, from pH 7 to pH 10, the rate of inactivation of the yeast and acid-fast organisms decreased. The inactivation of the yeast (C. *parapsilosis*) decreased significantly at the higher pH, i.e., with a lower concentration of hypochlorous acid; the acid-fast organism (M. *fortuitum*) was not affected to the same degree. However, it may be concluded that yeasts and acid-fast organisms are more effectively inactivated by hypochlorous acid than hypochlorite ion. No conclusions can be drawn concerning E. *coli* since it could not be successfully enumerated at either pH due to their high degree of inactivation under all experimental conditions.

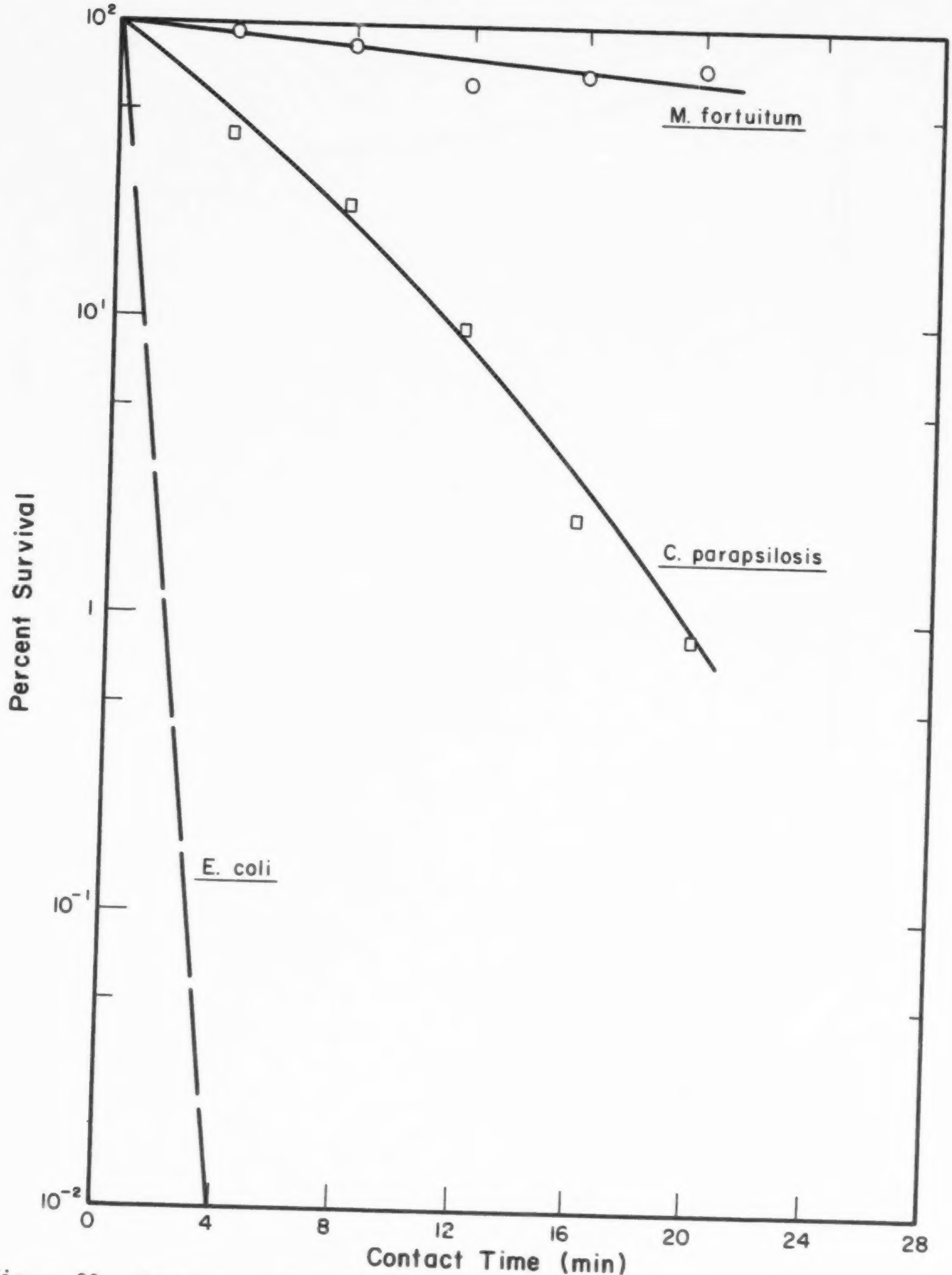


Figure 23. Continuous Flow Inactivation Studies with 0.53 mg/ℓ Free Available Chlorine Residual Using Mixed Pure Cultures in Chlorine Demand Free Buffer at pH 7 and 22.5°C

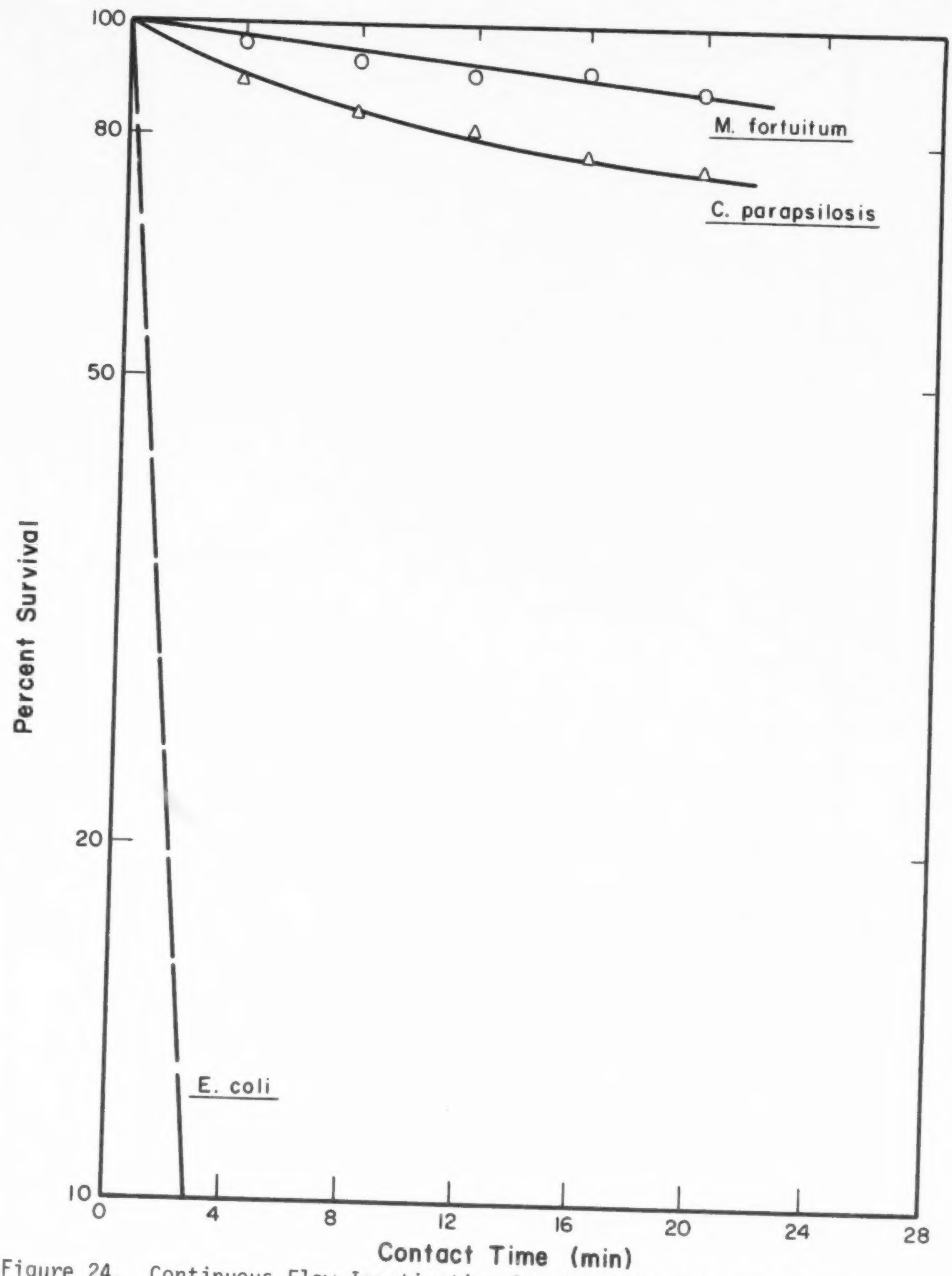


Figure 24. Continuous Flow Inactivation Studies with 0.96 mg/ℓ Free Available Chlorine Residual Using Mixed Pure Cultures in Chlorine Demand Free Buffer at pH 10 and 24.5°C

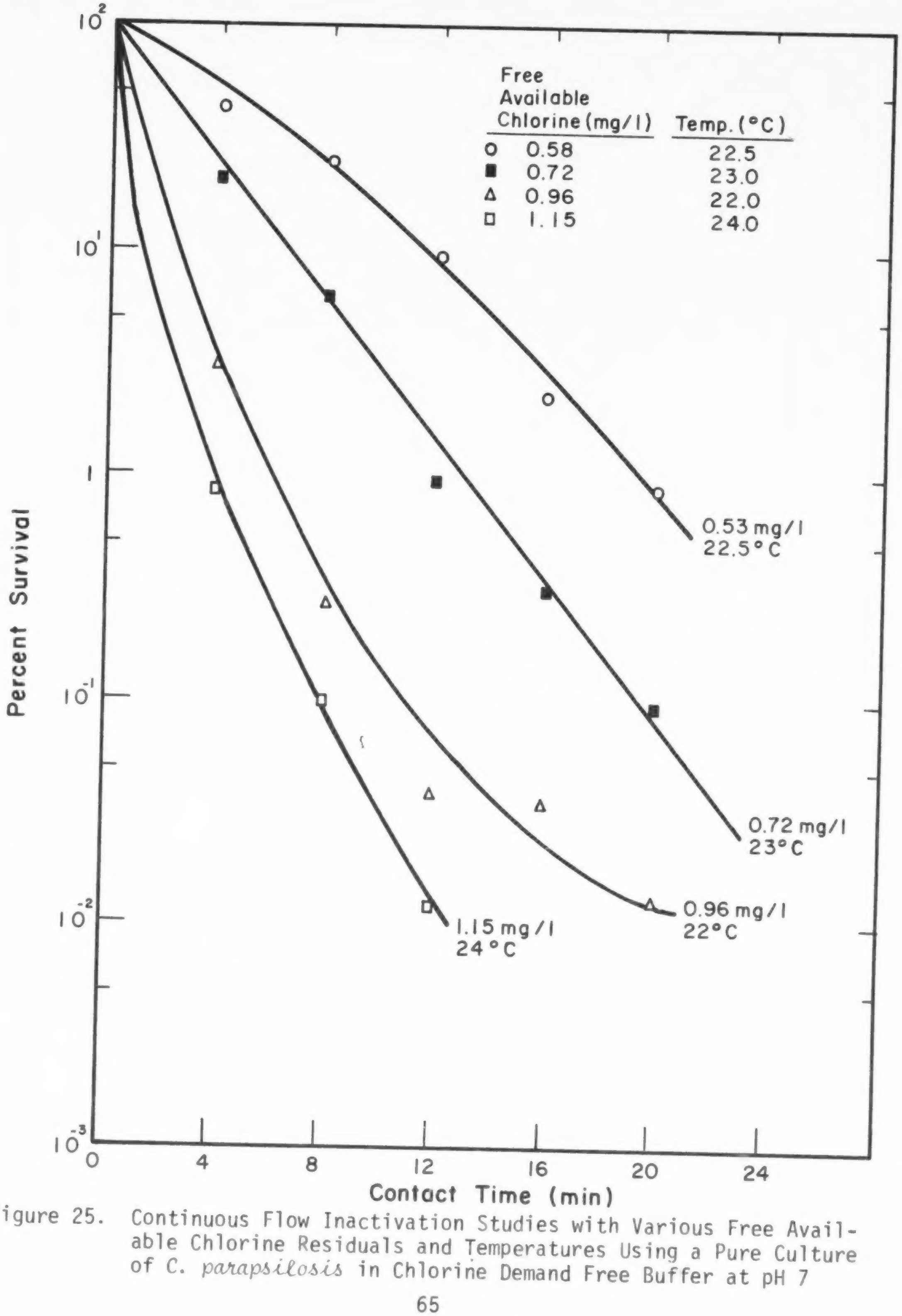


Figure 25. Continuous Flow Inactivation Studies with Various Free Available Chlorine Residuals and Temperatures Using a Pure Culture of C. *parapsilosis* in Chlorine Demand Free Buffer at pH 7

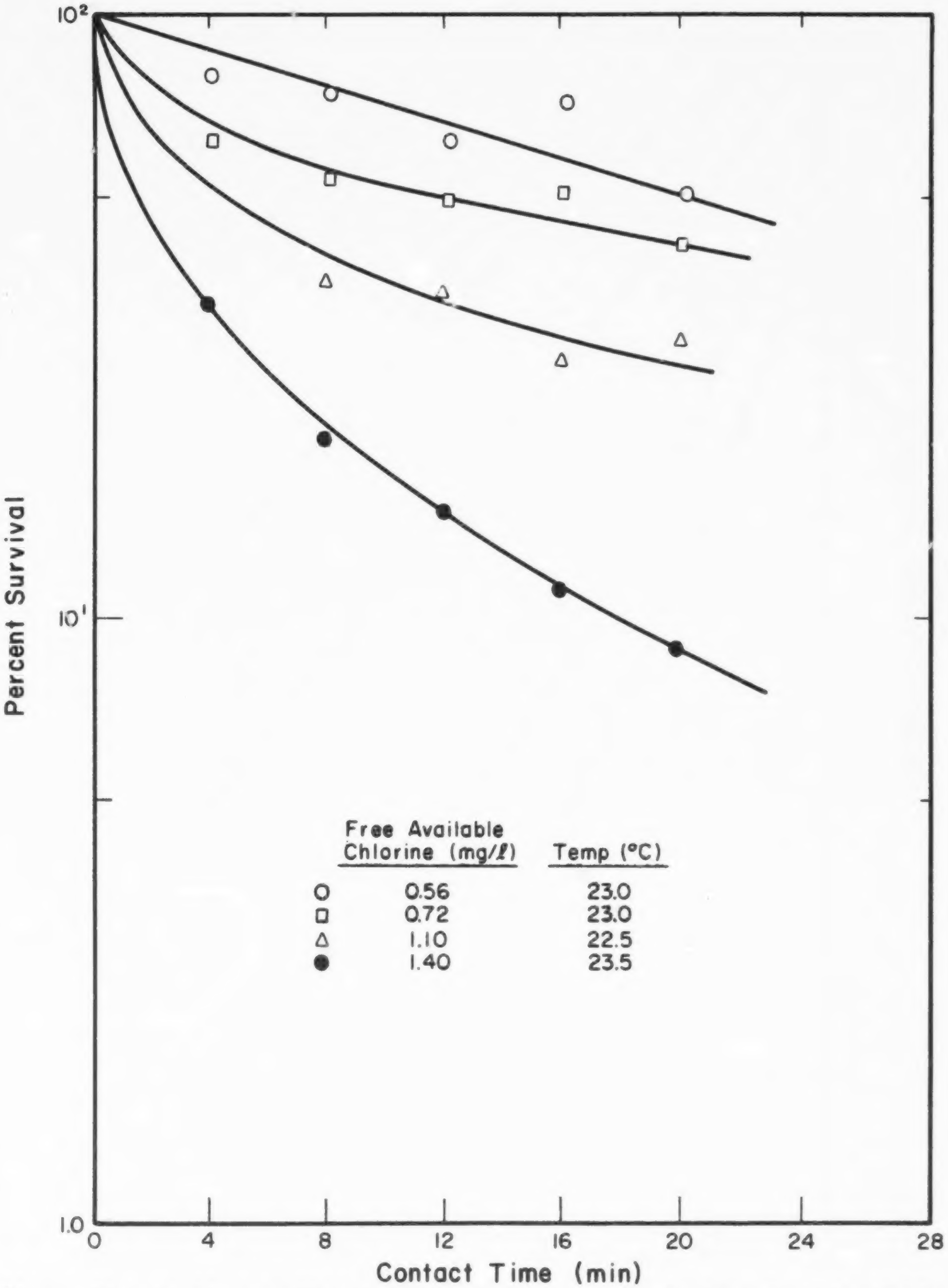


Figure 26. Continuous Flow Inactivation Study with Various Free Available Chlorine Residuals and Temperatures Using a Pure Culture of *M. fortuitum* in Chlorine Demand Free Buffer at pH 7

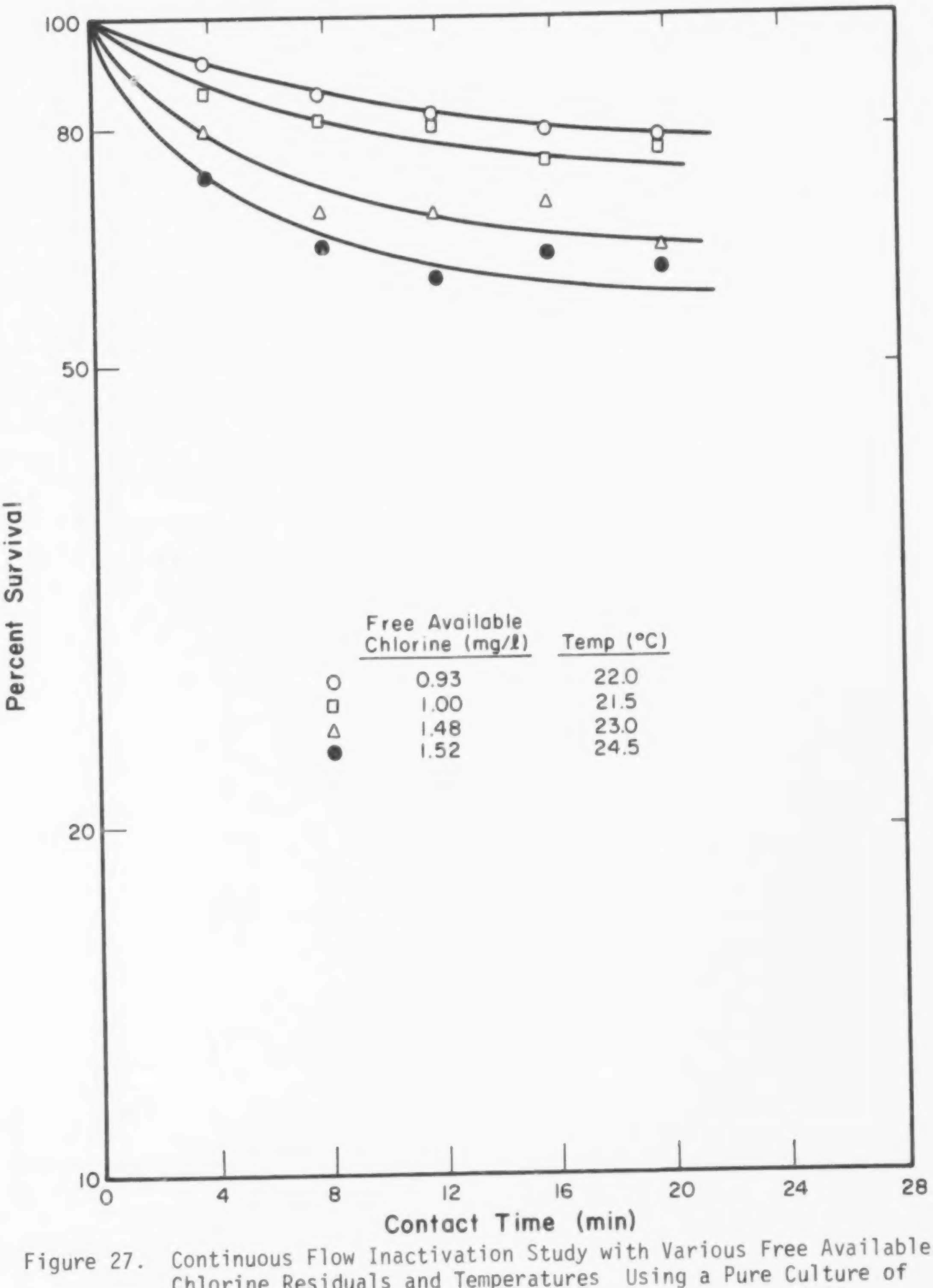


Figure 27. Continuous Flow Inactivation Study with Various Free Available Chlorine Residuals and Temperatures Using a Pure Culture of *C. parapsilosis* in Chlorine Demand Free Buffer at pH 10

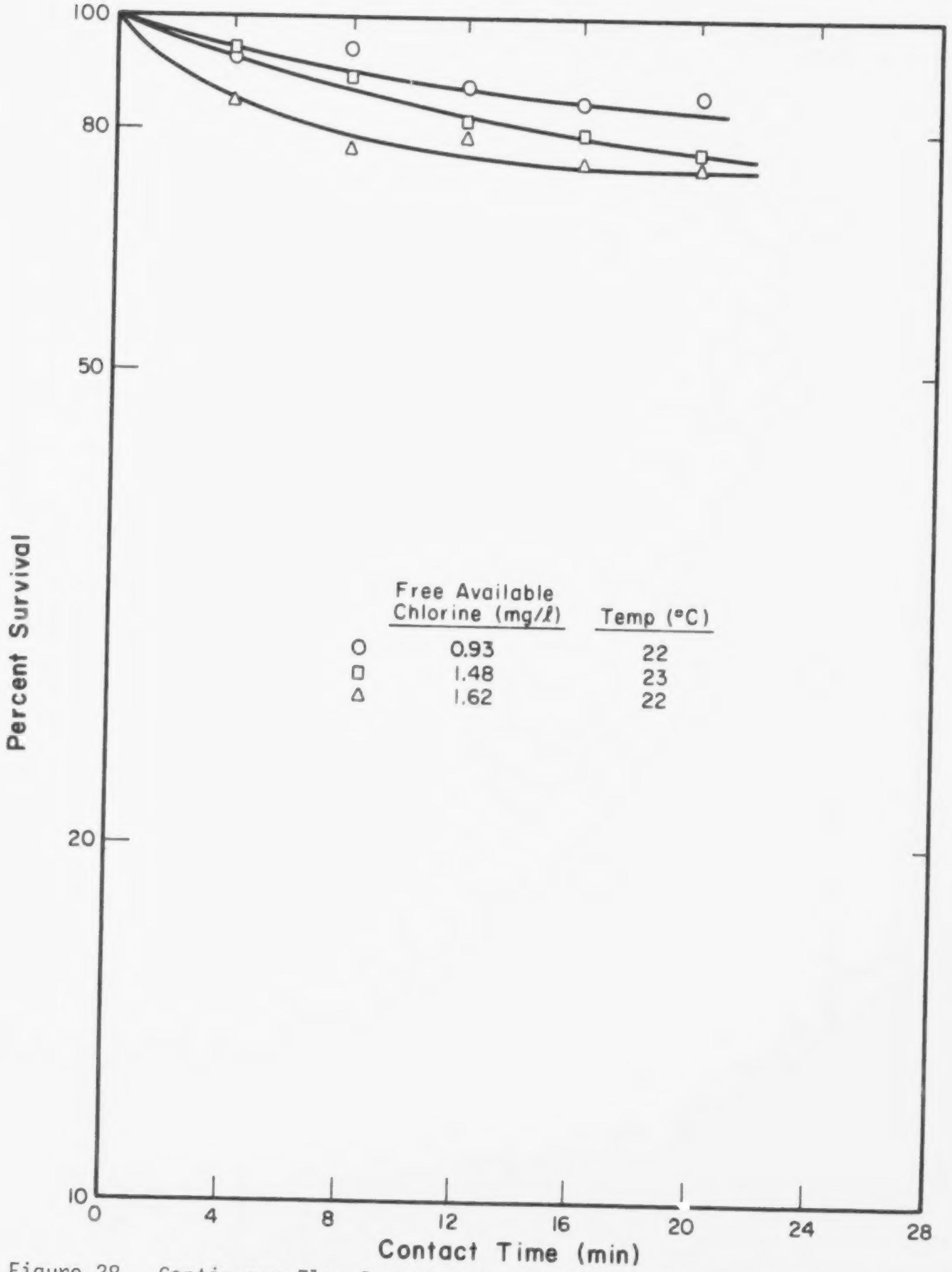


Figure 28. Continuous Flow Inactivation Study with Various Free Available Chlorine Residuals and Temperatures Using *M. fortuitum* in Chlorine Demand Free Buffer at pH 10

Comparison of these observations with those of the batch reactor studies with free chlorine show general agreement (5). In both studies, acid-fast organisms were found to be more resistant than yeasts, which in turn were more resistant than the coliform organisms to free available residual chlorine under similar experimental conditions. This was true for experiments at both pH 7 and pH 10, with the rate of inactivation of the yeast and acid-fast organisms always being less at the higher pH value. The response of the yeast and acid-fast organisms to the higher pH value was similar in these studies as in the batch reactor studies. In the batch reactor studies it was calculated that in the inactivation of the yeast, hypochlorous acid was approximately 5-20 times more effective than the hypochlorite ion. In the case of the acid-fast organism, it was found that hypochlorous acid was only 1.1-2.5 times more effective than the hypochlorite ion (5). Considering the results of the continuous flow experiments, it was observed that in the inactivation of the yeast and acid-fast organisms, the hypochlorous acid concentration at pH 7 was approximately 4-10.5 and 1.5-3 times more effective, respectively, than the hypochlorite ion which predominates at pH 10. Therefore, both the continuous flow and batch reactor studies support the conclusion that hypochlorous acid is a more effective disinfectant for inactivation of yeasts and acid-fast organisms than hypochlorite ion.

A comparison of the *E. coli* data from the two studies is of questionable significance based on the fact that in the batch studies the *E. coli* suspensions were prepared from nutrient agar slants and were enumerated as fecal coliforms, whereas in these continuous flow studies, *E. coli* suspensions were prepared from nutrient broth cultures and were enumerated as total coliforms. Although these differences exist, it may be concluded that there was general agreement in the inactivation of *E. coli* between the two studies in comparing the experiments performed at pH 7. In neither study was it possible to reliably demonstrate the presence of *E. coli*, even with the shortest contact time, due to their high degree of inactivation. With the batch reactor studies, more than 3 logs reduction of *E. coli* occurred in 4 min, while these continuous flow studies showed greater than 4 logs inactivation with the same contact time. In the batch reactor studies at pH 10, up to 1.8 percent of *E. coli* survived 1.0 mg/ℓ free available residual chlorine after a contact time of 10 min; inactivation of *E. coli* in the continuous flow system amounted to more than 4 logs in 4 min. The reason for this discrepancy is not apparent.

Natural Population Studies

The natural population studies were performed as either breakpoint or non-breakpoint chlorination experiments. In all cases, the test solution in each inactivation experiment was a grab sample of the clarified activated sludge effluent from the East Side Wastewater Treatment Plant, Sanitary District of Urbana-Champaign. A review of the operating and performance characteristics for the activated sludge unit of the treatment plant, as supplied by the Sanitary District, together with additional data (TOC and COD), indicated that the plant was achieving satisfactory results with respect to removal of organic matter and suspended solids. Considering all the grab samples of activated sludge effluent analyzed and used in the natural population study, the average BOD_5, TOC, COD, and suspended solids in the effluent was found to be 14.3, 21.5, 77.5, and 9.0 mg/ℓ, respectively. The pH varied from 7.30 to 7.76 while the ammonia concentration in the effluent averaged 5.35 mg/ℓ as N. A statistical analysis was performed to determine if there was any correlation between inactivation of the test organisms and the physical/chemical characteristics of the test solution. Using a step-wise regression, it was determined that the independent variable of chlorine residual accounted for greater than 85 percent of the variance in the inactivation of each of the three test organisms.

In the non-breakpoint chlorination experiments, the dosage of chlorine approximated that used in practice when chlorinating a wastewater effluent. The chlorine dose varied from 1.5-4.5 mg/ℓ and the corresponding reactor effluent residual chlorine concentration, measured as total combined chlorine, varied from 0.48-2.6 mg/ℓ. In these experiments, the reactor effluent pH and temperature varied between 6.9 and 7.3 and 22° and 24°C, respectively.

Results of the non-breakpoint natural population chlorination studies, based upon the inactivation of acid-fast, yeast, and fecal coliform organisms by various levels of combined residual chlorine, are shown in Figures 29 through 32. Under all conditions studied in these experiments, the acid-fast organisms consistently showed the lowest inactivation rate, followed by that of the yeasts; the fecal coliform organisms always exhibited the highest rate of inactivation. In general, for a contact time of 20 min, the overall reduction in acid-fast organisms was slightly less than 1 log, while the yeast showed a reduction greater than 1 log; approximately 3.5 log reduction was observed with the fecal coliform organisms. Some variation in inactivation occurred for all three groups of organisms, depending on the experimental conditions. As seen from these figures showing the results of chlorinating a natural population of organisms such as might be found in an activated sludge effluent, the acid-fast and yeast organisms were consistently more resistant to combined available chlorine than the coliform organisms.

These plots also show that as the chlorine dosage and resulting residual increased, the inactivation of the test organisms also increased. This expected increase in inactivation with increased combined chlorine residual was not as pronounced in the current study as in the study with chloramines reported earlier (5). In the current natural population study, chlorine was added as free chlorine which then reacted with the ammonia to form combined chlorine, while the chloramine study was done with preformed chloramines. It may be hypothesized that organism inactivation under the former conditions should be greater than that observed in the chloramine experiments, because of the initial presence of free available chlorine.

The results of the current study also agree with the data presented earlier in this report for the inactivation of the test organisms at the St. Joseph Wastewater Treatment Plant. Under similar chlorination conditions, the St. Joseph study showed a mean log reduction, due to chlorination of secondary effluent, of 0.40, 1.3, and 3.2 logs for the acid-fast, yeas

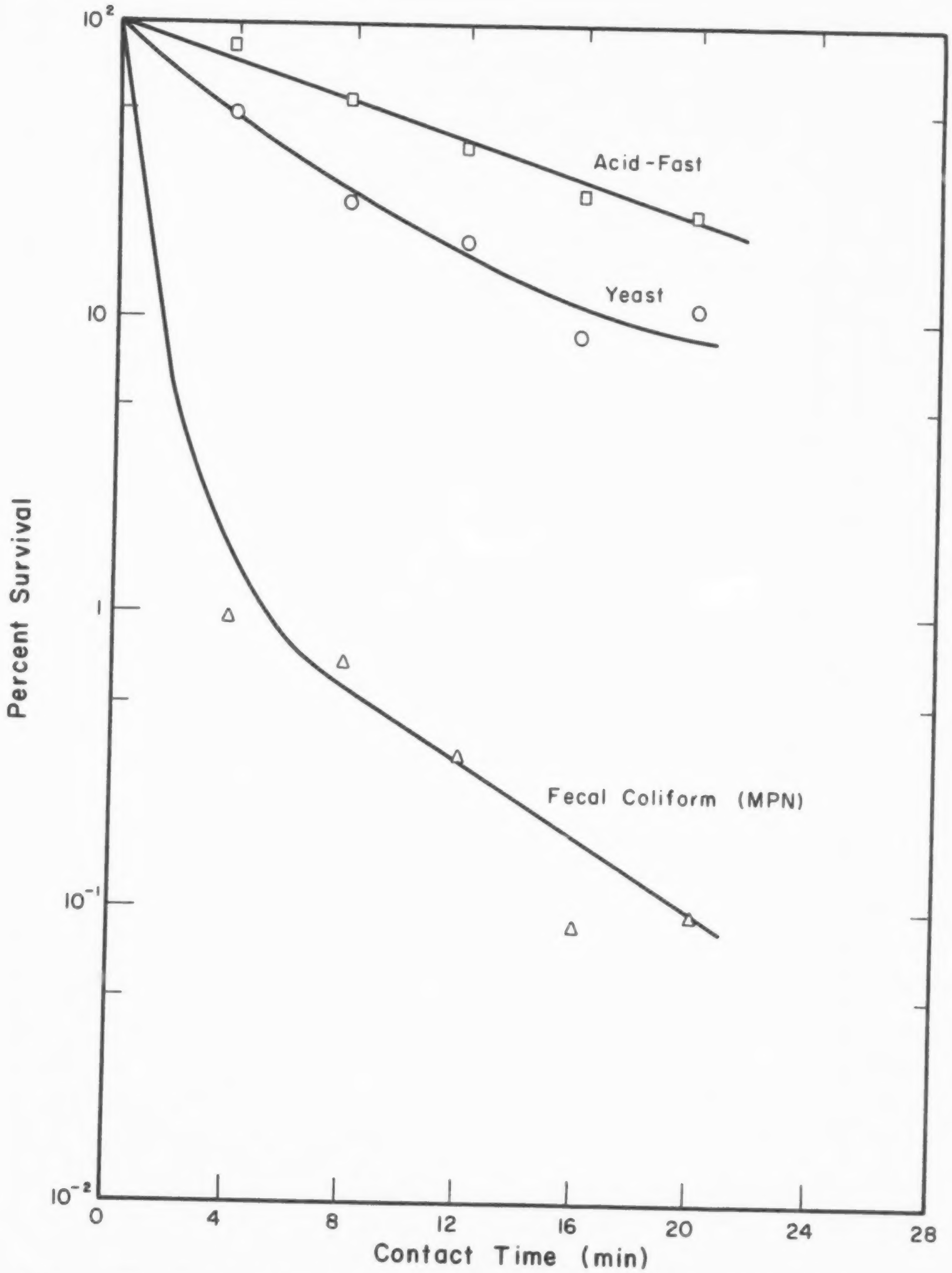


Figure 29. Non-Breakpoint Chlorination Study with 0.63 mg/ℓ Total Combined Residual Using Clarified Activated Sludge Effluent

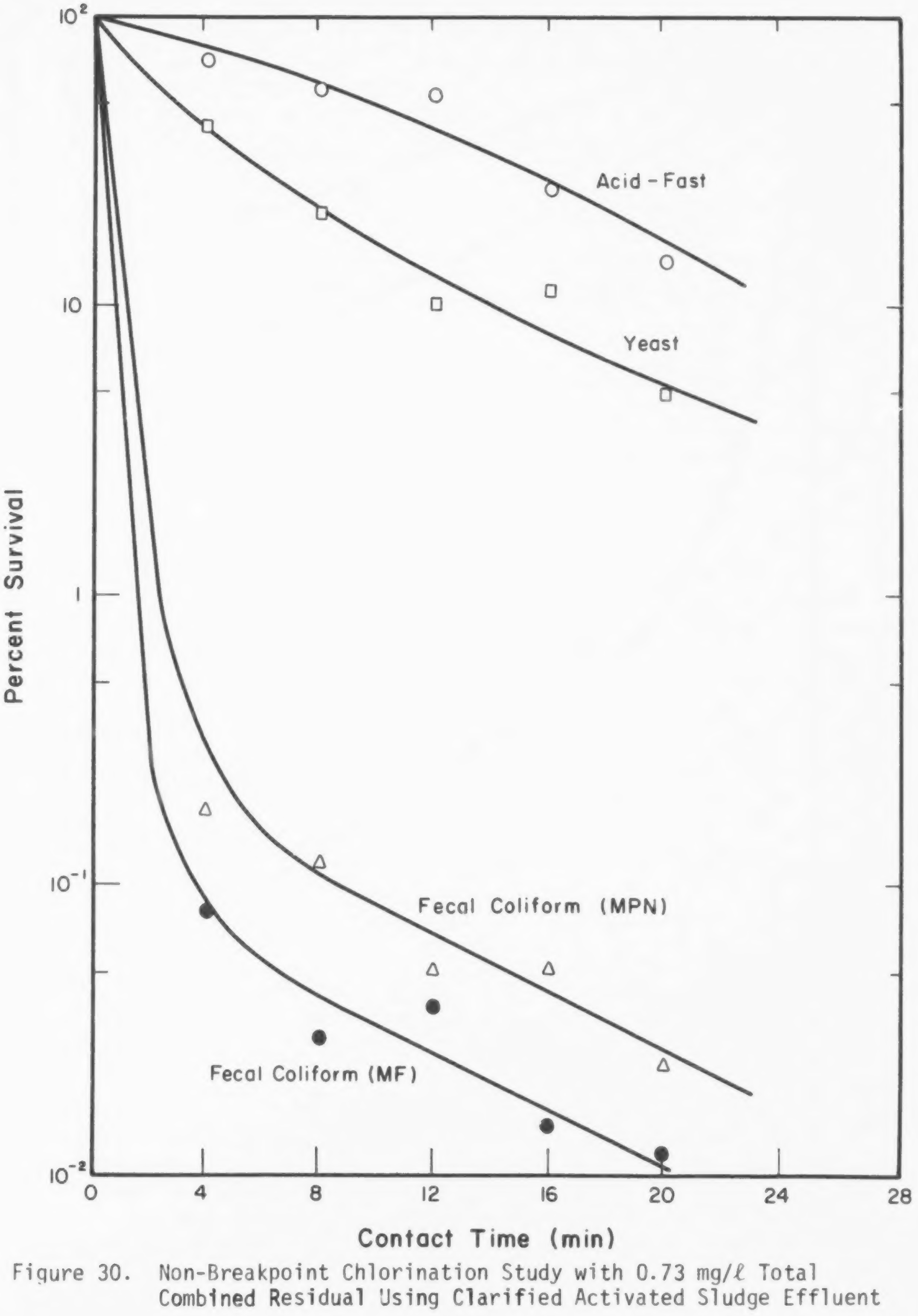


Figure 30. Non-Breakpoint Chlorination Study with 0.73 mg/ℓ Total Combined Residual Using Clarified Activated Sludge Effluent

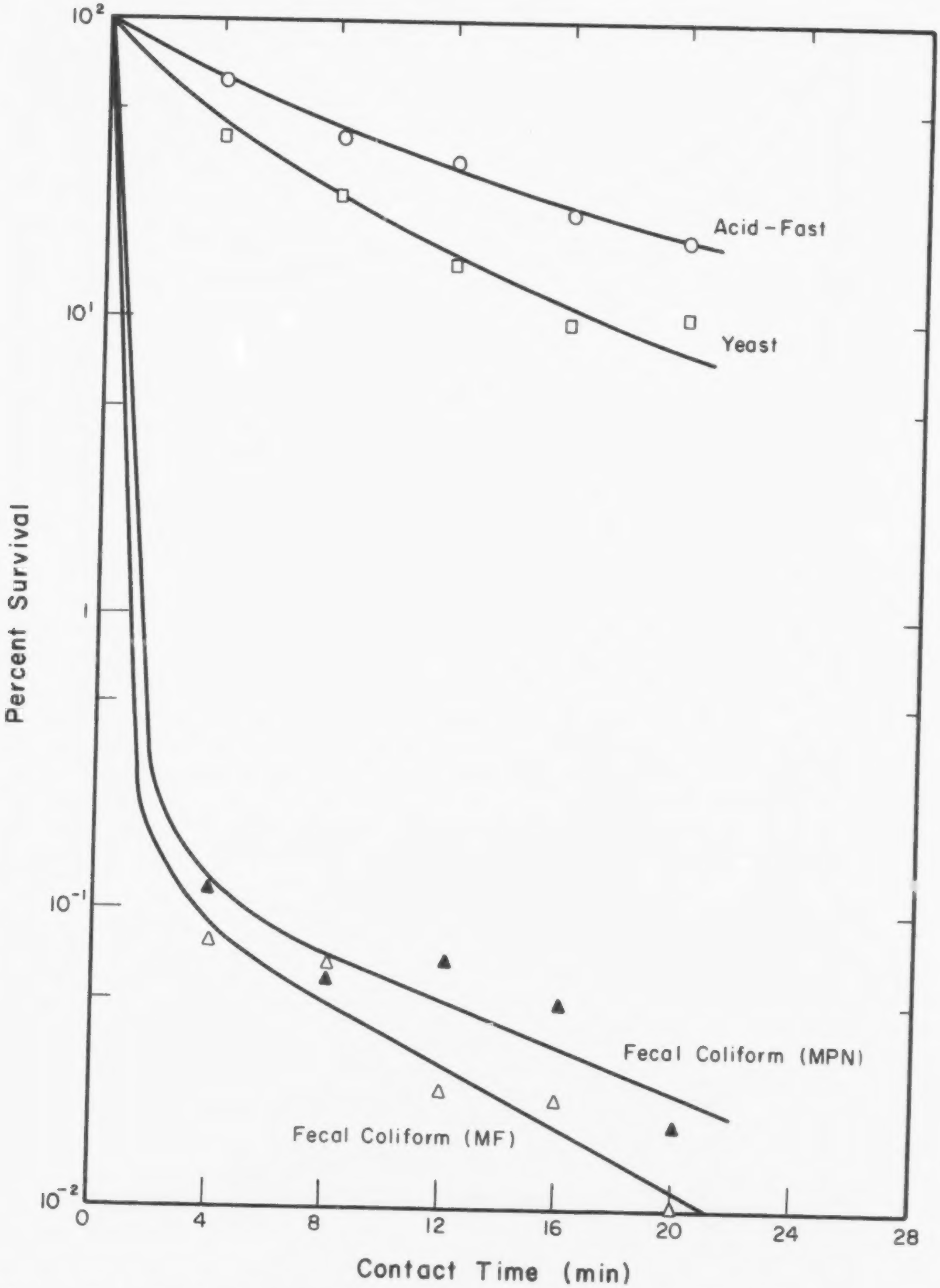


Figure 31. Non-Breakpoint Chlorination Study with 0.84 mg/ℓ Total Combined Residual Using Clarified Activated Sludge Effluent

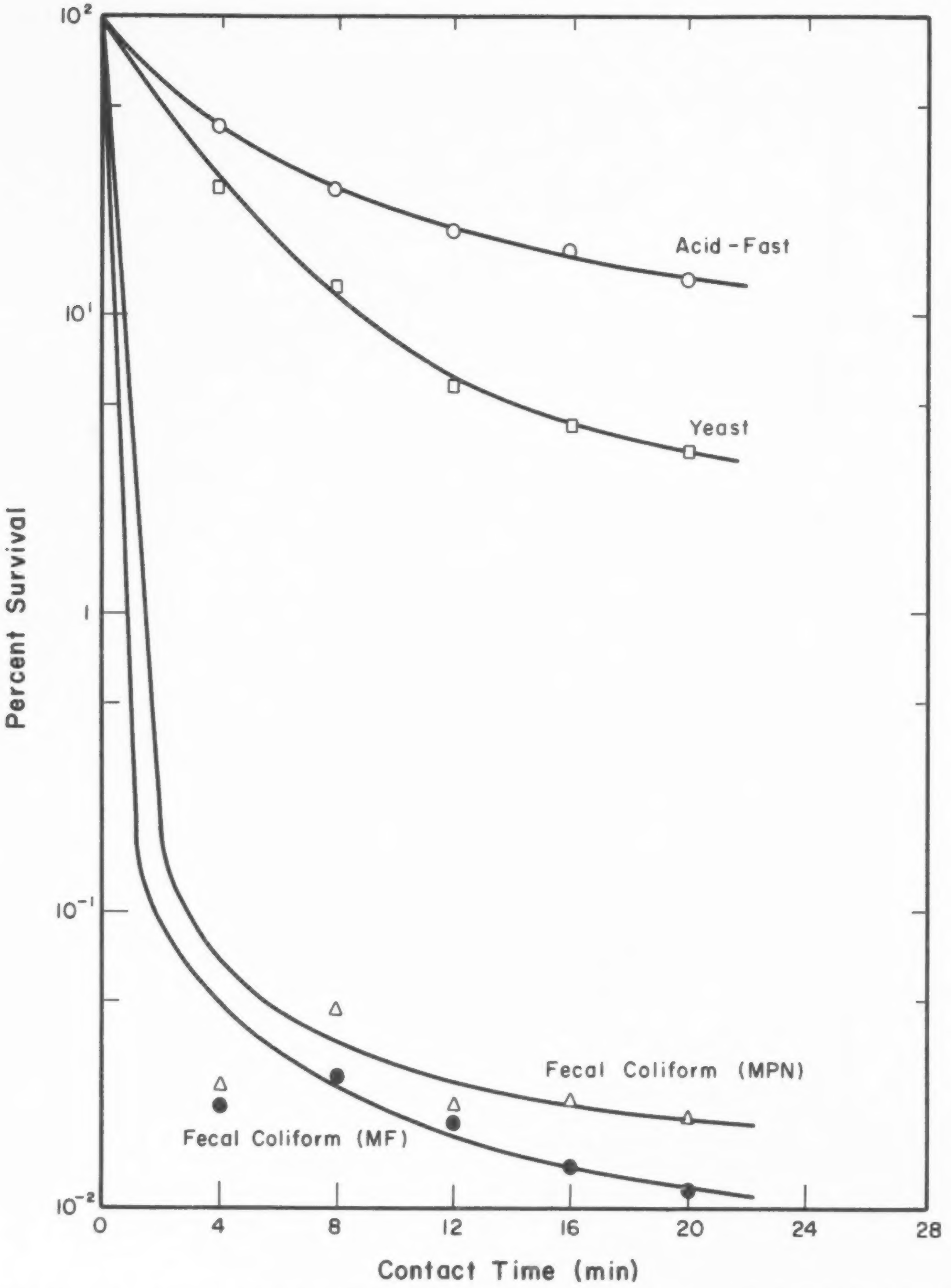


Figure 32. Non-Breakpoint Chlorination Study with 1.73 mg/ℓ Total Combined Residual Using Clarified Activated Sludge Effluent

and fecal coliform organisms, respectively, which compares favorably with the inactivation of the same organisms in the current study.

Experimental results for studies in which fecal coliforms were enumerated by both the MPN and MF techniques are presented in Figures 30, 31, and 32. In these experiments, the MPN technique always gave a higher recovery of fecal coliforms than the MF procedure. The densities of fecal coliforms, as determined by the MF procedure, agree with the range of fecal coliform densities found in activated sludge effluents in past studies (5).

The breakpoint chlorination experiments were performed so as to obtain a free available residual of approximately 0.5-1.0 mg/ℓ in the reactor effluent. A sufficient volume of activated sludge effluent was collected so that experiments at the two different levels of free available residual chlorine could be made with the same grab sample. For the breakpoint studies, the chlorine dosage varied from approximately 63-87 mg/ℓ, whereas the free available residual chlorine concentration varied from 0.45-1.10 mg/ℓ. Traces of combined chlorine residual were detected in the reactor effluent on occasion, but it could not be measured with any accuracy or consistency and, thus, was not considered valid. The reactor effluent pH and temperature for the breakpoint experiments varied from 7.15-7.35 and 18°-21°C, respectively.

Results from the breakpoint chlorination experiments were limited due to the high degree of inactivation that occurred for each group of organisms studied. With the experimental arrangement used, neither the yeasts nor the coliform organisms could be accurately enumerated under any of the experimental conditions studied. The acid-fast organisms, on the other hand, were successfully enumerated in several of the breakpoint experiments, but only with the shorter contact times of 4 and 8 min, and in the reactor effluent (contact time, 20 min) when a large sample volume could be collected and filtered.

Table 17 presents the chlorine dosage and resultant residual of each breakpoint experiment, and the log reduction for each organism group measured after a 20 min contact time. Of the three organism groups studied, the acid-fast organisms were the most resistant to chlorination. The inactivation results for the yeast and coliform organisms do not permit a definite conclusion to be reached regarding their relative resistance to breakpoint chlorination.

The survival curves in Figure 33 for the acid-fast organisms are given for the breakpoint experiments in which the acid-fast organisms were successfully enumerated. Based upon these data, it would appear that initially, within approximately 4 min contact time, there was a very rapid inactivation of the acid-fast organisms; this was then followed by a much slower rate of inactivation. This observation is consistent with the characteristics associated with members of the acid-fast group of organisms and their variable resistance to chlorination. The natural population of acid-fast organisms in wastewater, i.e., activated sludge effluent, probably includes species with varying degrees of sensitivity to chlorination, e.g., M. *phlei* is more

TABLE 17. INACTIVATION DATA FOR BREAKPOINT CHLORINATION EXPERIMENTS

Date	Chlorine dosage, mg/ℓ	Free chlorine residual, mg/ℓ	Log reduction* (20 min contact time)		
			Coliforms	Yeasts	Acid-Fast Organisms
11/28	63.0	0.59	>4.70	>2.24	2.16
	65.0	1.04	>4.56	>2.40	2.26
12/06	86.0	0.45	>3.63	>1.86	>1.96
	87.0	1.10	>3.84	>1.98	>1.77
12/17	78.0	0.46	>4.03	>2.17	2.13
	81.0	0.95	>3.88	>2.25	>2.29

*Because of enumeration difficulties, much of the data must be expressed as greater than (>).

sensitive to chlorination than M. *fortuitum* (5). Under the severe conditions of breakpoint chlorination, it might be expected that the more sensitive or a percentage of the total population of acid-fast organisms would be initially inactivated at an apparent high rate while the more resistant species would be inactivated at a slower rate.

In the previous chloramine study (5) using a contact time of 20 min, M. *fortuitum* showed approximately 0.3 log reduction when subjected to 3.25 mg/ℓ of chloramine, while C. *parapsilosis* exhibited approximately 0.52 log reduction with 2.83 mg/ℓ of chloramine. In the current breakpoint study, the initial density of M. *fortuitum* was reduced by 0.7 log and C. *parapsilosis* showed greater than 4 logs reduction when both were exposed to 1.15 mg/ℓ of free available residual chlorine for 20 min. Considering the non-breakpoint chlorination study, a chlorine dosage of 4.0 mg/ℓ which produced a residual of 1.73 mg/ℓ of total combined chlorine resulted in approximately 0.83 and 1.46 log reduction of the acid-fast and yeast organisms, respectively.

Care must be taken in making any direct comparison of the results of the natural population study with those of the other studies mentioned. The activated sludge used in the natural population study probably contained several members of each test organism group, members which are known to show a variable response to chlorine disinfection. In contrast, the free chlorine and chloramine studies used a known chlorine resistant member of each organism group. The presence of other organisms of these groups in the secondary effluent used in the natural population study, which may have been less resistant to chlorination, may be the reason for the observed discrepancy between the expected and observed inactivation of the test organisms among the three studies.

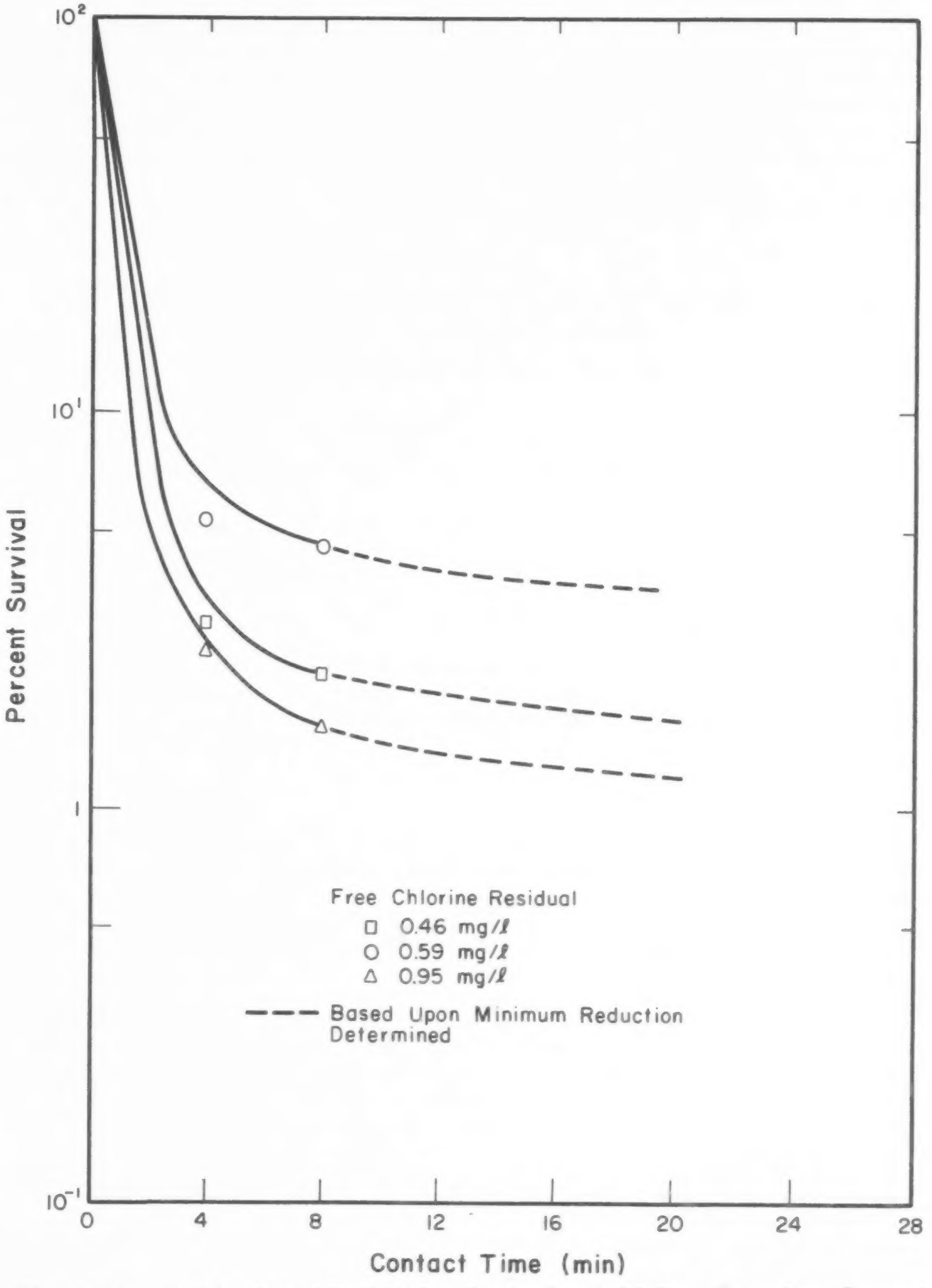


Figure 33. Breakpoint Chlorination Study for Acid-Fast Organisms Present in Clarified Activated Sludge Effluent

Another series of chlorine inactivation experiments was performed with a natural population of organisms using activated sludge effluent which was subsequently treated by activated carbon. The measured physical and chemical characteristics of the activated carbon column influent, i.e., clarified activated sludge, and effluent are given in Table 18. Comparison of the influent and effluent values presented in Table 18 shows that only TOC was significantly affected by treatment with activated carbon, i.e., 55 to 75 percent reduction in TOC.

TABLE 18. PHYSICAL AND CHEMICAL CHARACTERISTICS OF ACTIVATED CARBON COLUMN INFLUENT* AND EFFLUENT

Exper. No.	pH		Temp, °C		Turbidity NTU		Suspended Solids mg/ℓ		TOC, mg/ℓ	
	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.
1	7.40	7.20	20.5	20.5	12.5	10.5	6.5	3.0	21.0	5.5
2	7.35	7.30	21.0	21.0	7.5	6.0	11.0	9.0	29.0	13.0
3	7.50	7.38	20.0	20.0	9.0	8.0	13.5	12.5	17.5	6.6

*Influent - clarified activated sludge effluent, East Side Wastewater Treatment Plant, Sanitary District of Urbana-Champaign

Experimental data and inactivation results of the chlorination experiments performed using the activated carbon treated secondary effluent are shown in Table 19. A slight reduction in the densities of all three test organisms occurred as a result of activated carbon treatment. In no case, however, was the reduction in organism density through the activated carbon column significant.

The actual chlorine inactivation results shown in Table 19 agree in general with the results of the previous natural population chlorination study. Acid-fast organisms were the most resistant of the three test organisms to chlorination, with the yeast being less sensitive to chlorination than the fecal coliform organisms. Comparison of the disinfection results as given in Table 19 for activated carbon treated secondary wastewater with those of the non-breakpoint natural population study shows that the acid-fast and fecal coliform organisms were inactivated to about the same degree in the two studies. On the other hand, the yeast organisms appear to be less chlorine resistant in the study utilizing activated carbon treated effluent than in the non-breakpoint experiments. Table 20 compares the log reductions of each of the three test organisms in the two studies

TABLE 19. RESULTS OF CONTINUOUS FLOW CHLORINATION INACTIVATION OF ORGANISMS PRESENT IN CLARIFIED ACTIVATED SLUDGE EFFLUENT AFTER TREATMENT BY ACTIVATED CARBON

Exper. No.	Chlorine dosage mg/ℓ	Total combined chlorine residual mg/ℓ
1	1.5	0.66
2	3.3	1.69
3	2.5	1.31

Exper. No.	Organism density in clarified activated sludge effluent No./100 mℓ	Organism density in activated carbon column effluent* No./100 mℓ	Organism density in chlorination reaction chamber effluent** No./100 mℓ
Acid-Fast			
1	1.09×10^3	1.036×10^3	2.07×10^2
2	6.23×10^3	6.105×10^3	9.16×10^2
3	2.7×10^3	2.6×10^3	2.7×10^2
Yeasts			
1	4.8×10^3	4.5×10^3	2.4×10^2
2	9.4×10^3	8.366×10^3	1.924×10^2
3	6.85×10^3	6.23×10^3	2×10^2
Fecal Coliforms			
1	8.6×10^5	7.998×10^5	5.6×10^2
2	5.53×10^5	5.31×10^5	1.274×10^2
3	6.9×10^5	6.66×10^5	1.465×10^2

*Effluent from the activated carbon column was collected and used as the influent organism test solution to the chlorination reactor chamber.

**Contact time 20 min.

TABLE 20. COMPARISON OF CHLORINE INACTIVATION RESULTS USING CLARIFIED ACTIVATED SLUDGE EFFLUENT AND ACTIVATED CARBON TREATED ACTIVATED SLUDGE EFFLUENT FOLLOWING 20 MINUTES CONTACT TIME

Total combined chlorine residual mg/ℓ	Activated carbon treatment, log reduction			Total combined chlorine residual mg/ℓ	Non-breakpoint chlorination log reduction		
	acid-fast	yeast	fecal coliform		acid-fast	yeast	fecal coliform
0.66	0.699	1.274	3.155	0.63	0.634	1.11	3.20
1.31	0.984	1.490	3.658	1.20	0.894	1.32	3.59
1.69	0.824	1.638	3.620	1.73	0.901	1.50	3.68

under similar conditions of chlorine residual. In general, it might be thought that, due to the lower TOC level in the chlorination experiments utilizing activated carbon treated effluent, a higher degree of inactivation of the test organisms would be achieved than in the non-breakpoint experiments because of the lower chlorine demand in the former. However, this does not appear to be the case, and in fact the opposite is true for the yeast organisms.

REMOVAL OF INDICATOR ORGANISMS BY CHEMICAL COAGULATION WITH ALUM, FERRIC CHLORIDE AND LIME

The major emphasis of these laboratory experiments was to evaluate the removal of a natural population of acid-fast organisms, yeasts and total coliforms by chemical coagulation when the conditions for turbidity removal were optimum. In order to define the conditions for optimum turbidity removal, each test water was subjected to a series of four experiments: 1) optimization of coagulant dose; 2) optimization of pH at the optimum coagulant dose; 3) optimization of flocculation time at a constant flocculation speed of 25 rpm; and 4) optimization of flocculation speed at a constant flocculation time of 20 min.

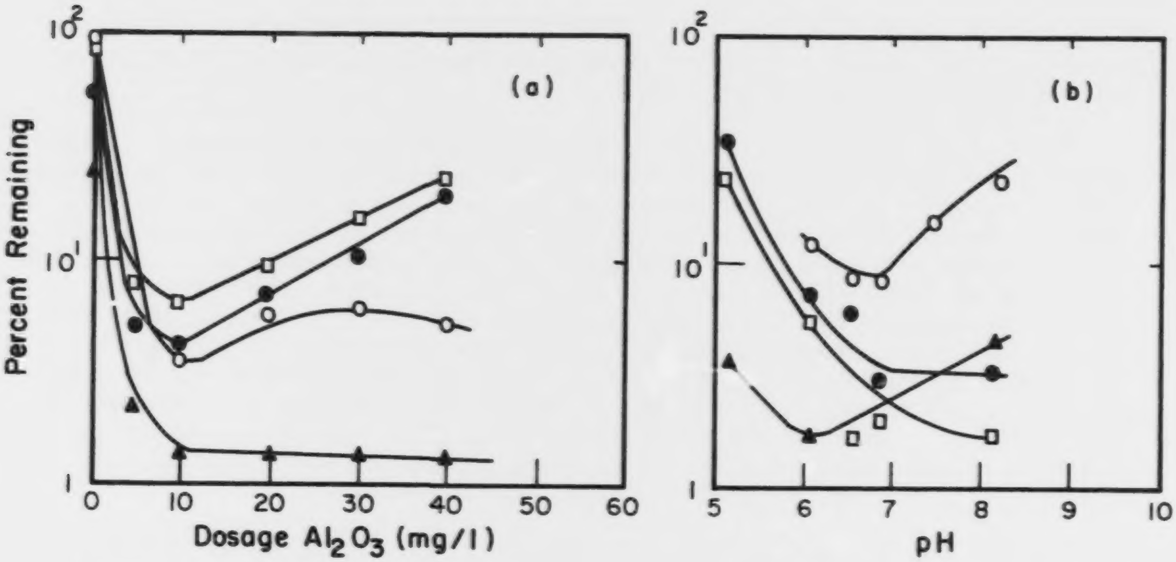
Each coagulant, alum, ferric chloride, and lime, was evaluated with two different test waters. The first water used consisted of dechlorinated tap water to which a stock suspension of kaolinite clay was added as a source of turbidity and a 1 percent volume of raw municipal wastewater was added as an organism inoculum. The second water was obtained from the Salt Fork of the Vermillion River in Vermillion County, Illinois. In this water, no organism inoculation was necessary because a natural population of the indicator organisms to be studied was present. Seven series of coagulation experiments were performed in all. Three of these used alum as a coagulant, and two each used ferric chloride and lime.

Figure 34 shows the results of a typical series of experiments. This particular series (experimental series 1) was performed with alum and dechlorinated tap water with kaolinite clay and raw wastewater added. As may be seen in Figures 34(a) and 34(b), the optimum removal of turbidity occurred with a dosage of 10 mg/ℓ alum as Al_2O_3 at a pH of approximately 7. Effective flocculation times and flocculation speeds showed a wide range of values. It may be observed in Figure 34(c) that with a flocculation speed of 25 rpm, stable turbidity removals of 96-97 percent were achieved with mixing times of between 20 and 50 min. Figure 34(d) shows that with a flocculation time of 20 min, all mixing rates between 25 and 50 rpm gave turbidity removals of 96-97 percent. The wide range of flocculation times and mixing speeds at which it was possible to produce a constant removal of turbidity indicate the stability of the floc particle to mixing stresses.

Since emphasis was given to the removal of the indicator organisms when the conditions for turbidity removal were optimum, more consideration will be given to the results in Figures 34(c) and 34(d) than those in Figures 34(a) and 34(b). Organism removals are reported as the range of experimental values observed throughout the range of optimum turbidity removal seen in the experiments performed to determine the optimum flocculation time and mixing speed. For example, Figure 34(c) shows that the least removal of yeasts occurred at a flocculation time of 5 min with a flocculation mixing rate of 25 rpm. The removal of yeasts was approximately 40 percent. Figure 34(d) shows that the greatest removal of yeast (98-99 percent) occurred with a mixing speed of 25 rpm with a flocculation time of 20 min. Therefore, a realistic estimate of yeast removal at the optimum conditions of turbidity removal would be a range of between 80 and 99 percent. A similar analysis of these figures indicates the range of acid-fast organism removal to be approximately 91-99.1 percent and the range of total coliform removal to be approximately 97-98 percent. The original turbidity in this series of experiments was 49 NTU. The original density of organisms, reported as number per 100 mℓ, was 2.6×10^4, 150, and 140 for total coliforms, acid-fast organisms, and yeasts, respectively.

Data obtained for the other six series of experiments were analyzed in a similar manner to that described for the results presented in Figure 34. The range of removal efficiency for each of the indicator group of organisms and turbidity, at optimum conditions for turbidity removal, are given in Table 21. Table 22 provides the physical-chemical conditions for optimum turbidity removal in all seven experimental series. Control densities of the indicator organisms (No/100 mℓ) and the initial turbidity in all seven series are shown in Table 23.

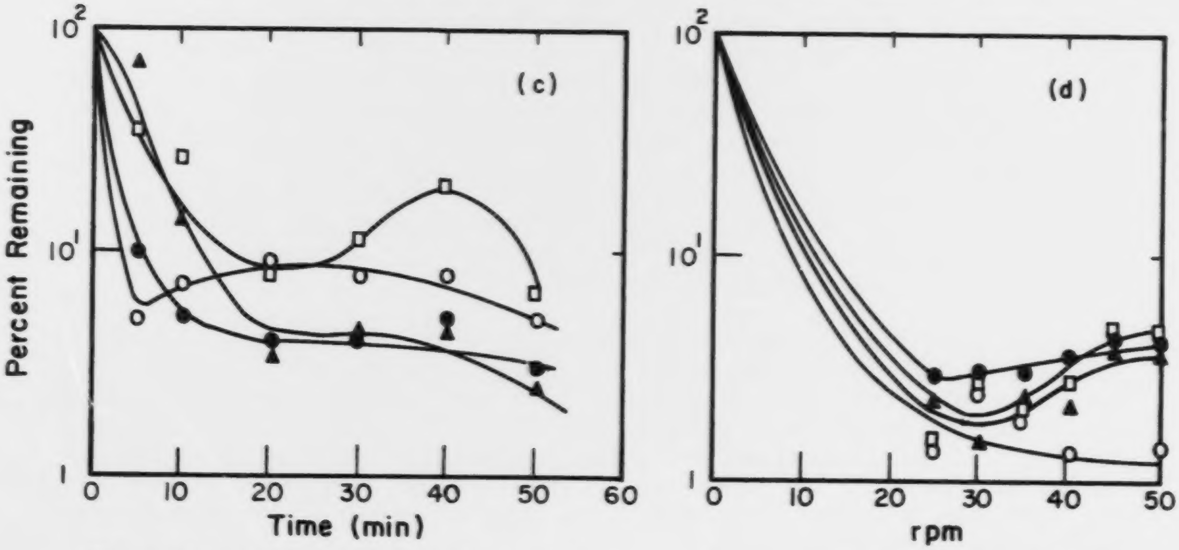
Experimental series 2 and 3 were also performed with alum. Series 2 was essentially the same as series 1 with the exception that the initial turbidity was 19 NTU rather than 49. The range of removal of the indicator organisms was essentially the same as in the first series, being approximately 90-99 percent for all three groups of indicator organisms when the



Dosage Optimization: pH 7, flocc. time = 20 min, flocc. rate = 30 rpm

pH Optimization: 10 mg/l Al_2O_3 flocc. time = 20 min, flocc. rate = 30 rpm

□ Y = yeast organisms
● T = turbidity
○ AF = acid-fast organisms
▲ TC = total coliform organisms



Flocculation Time Optimization: pH 7, 10mg/l Al O , flocc. rate = 25 rpm

Flocculation Speed Optimization: pH 7, 10 mg/l Al_2O_3, flocc. time = 20 min

Figure 34. Removal of Indicator Organisms and Turbidity with Alum (Tap Water, Kaolinite Clay, and 1.0 Percent Raw Wastewater; Temperature = 10-15°C)

TABLE 21. REMOVAL OF INDICATOR ORGANISMS AT OPTIMUM CONDITIONS FOR TURBIDITY REMOVAL

Experimental series	Turbidity, %	Total coliforms, %	Acid-fast organisms, %	Yeasts, %
1) Alum, kaolinite clay and 1% raw wastewater	96-97	97-98	91-99.1	80-99
2) Alum, kaolinite clay and 1% raw wastewater	80-98	93-99	89-93	89-98
3) Alum, river water	85-95	93-98	>93	92-98
4) Ferric chloride, kaolinite clay and 1% raw wastewater	95-99	98-99.5	>95	97-99
5) Ferric chloride, river water	70-98	60-97	>90	78-94
6) Lime, kaolinite clay and 1% raw wastewater	85-96	99.97-99.98	>99.8	98-99
7) Lime, river water	75-80	99.7-99.97	--	97-99.8

removal of turbidity was optimum. The range of optimum turbidity removal was found to be slightly higher (80-98 percent) than in the first series. This was due to the method of recording optimum results. The third experiment of the second series (optimization of flocculation time) showed a constant removal of turbidity from 97-98 percent with flocculation times of 20-50 min. The fourth experiment of the series (optimization of mixing speed) indicated a removal of turbidity of 80-85 percent with mixing speeds of 25-50 rpm. Hence, the range of turbidity removal recorded for the second series in Table 21 is the combined result of the two experiments and is given as the range between 80 and 98 percent.

Experimental series 3 was performed utilizing river water. In these

TABLE 22. OPTIMUM PHYSICAL-CHEMICAL CONDITIONS FOR OPTIMUM TURBIDITY REMOVAL

Experimental series	Coagulant dose mg/ℓ	pH	Flocculation time (min) at 25 rpm	Flocculation speed (rpm) for 20 min
1) Alum, kaolinite clay and 1% raw wastewater	10.0 as Al_2O_3	7.0	20-50	25-50
2) Alum, kaolinite clay and 1% raw wastewater	10.0 as Al_2O_3	7.0	20-50	25-50
3) Alum, river water	5.0 as Al_2O_3	7.0	30-50	30-50
4) Ferric chloride, kaolinite clay and 1% raw wastewater	30.0 as Fe_2O_3	9.6	30-50	30-50
5) Ferric chloride, river water	30.0 as Fe_2O_3	7.9	30-50	30-50
6) Lime, kaolinite clay and 1% raw wastewater	300 as $CaCO_3$	10.9	40-50	40-50
7) Lime, river water	500 as $CaCO_3$	10.7	40-50	35-50

experiments, the optimum removal of turbidity was found when 5.0 mg/ℓ alum as Al_2O_3 was used. Other considerations, such as pH, flocculation time and mixing speed, as well as organism and turbidity removal ranges were very similar to those recorded in series 1 and 2. In considering the first three series of experiments as a whole, it may be summarized that under optimum conditions for turbidity removal, approximately 90-99 percent removal of the turbidity and indicator organisms may be expected when alum was used as the coagulant.

Special consideration should be given to the data for the removal of acid-fast organisms reported for experimental series 3-7 (Table 21). In

TABLE 23. INITIAL CONDITIONS FOR THE EXPERIMENTAL COAGULATION

Experimental series	Turbidity NTU	Total coliforms No./100 mℓ	Acid-fast organisms No./100 mℓ	Yeasts No./100 mℓ
1) Alum, kaolinite clay and 1% raw wastewater	49	2.6×10^4	150	140
2) Alum, kaolinite clay and 1% raw wastewater	19	2.0×10^6	170	120
3) Alum, river water	19	1.8×10^3	230	95
4) Ferric chloride, kaolinite clay and 1% raw wastewater	23	1.5×10^5	20	125
5) Ferric chloride, river water	12	8.3×10^2	58	340
6) Lime, kaolinite clay and 1% raw wastewater	37	1.2×10^5	130	230
7) Lime, river water	19	4.7×10^3	--	170

series 3-6, removal ranges are given as "greater than" an indicated level of removal. No removal range is cited for the acid-fast organisms in series 7. A problem was encountered with a contaminating mold growth which inhibited the recovery of acid-fast organisms in some of the experiments. In series 3-6, only one of the two final optimum experiments in each series, i.e., optimization of flocculation time and optimization of mixing speed, yielded reliable results. The results of the reliable experiments are the recorded levels in Table 21. Data from the other experiments, i.e., those in which the acid-fast plates showed signs of contamination, tended to always show a higher removal of these organisms than the level recorded in Table 21. The range of removal efficiencies is therefore presented as "greater than" the reliable values obtained. Reliable results for acid-fast organism removal were not obtained in experimental series 7.

The results of the coagulation experiments using ferric chloride with tap water plus kaolinite clay and raw wastewater (experimental series 4) were very similar to those obtained with alum. Removal of turbidity and the indicator organisms was generally 95-99 percent. The removal of total coliforms reached as high as 99.5 percent in some experiments. Optimum conditions of turbidity removal in this series were a coagulant dose of 30.0 mg/ℓ as Fe_2O_3 and a pH of 9.6. The ranges of flocculation times and mixing speeds which produced optimum turbidity removal were somewhat less than with alum, being from 30-50 min and 30-50 rpm, respectively.

A much wider range of results was obtained with river water and ferric chloride than was reported for the series utilizing ferric chloride and the tap water suspension. With the river water, turbidity removal was 70-98 percent, total coliform removal was 60-97 percent, yeast removal was 78-94 percent, and the removal of acid-fast organisms was estimated to be greater than 90 percent (Table 21). Optimum chemical conditions for turbidity removal were a coagulant dose of 30.0 mg/ℓ as Fe_2O_3 and a pH of 7.9 (Table 22). Although flocculation time and mixing ranges were similar to those cited for turbidity removal in the tap water suspension, i.e., 30-50 min and 30-50 rpm, no river water turbidity removal was noted if the flocculation time was less than 30 min in the third test of the series, i.e., optimization of flocculation time with a mixing speed of 25 rpm.

Turbidity removal by lime precipitation showed the greatest dependence upon flocculation time and mixing speed compared to the other two coagulants. Ranges of flocculation time and mixing speeds producing optimum turbidity removal conditions were limited to 40-50 min and 40-50 rpm in the experiments with tap water plus kaolinite clay and raw wastewater, and 40-50 min and 35-50 rpm in the river water experiments (Table 22). Dosage requirements in these two series of tests were very critical. Failure to add sufficient lime caused an increase in turbidity. Optimum turbidity removal with the tap water suspension was accomplished with 300 mg/ℓ as $CaCO_3$ at pH 10.9 while 500 mg/ℓ lime as $CaCO_3$ at pH 10.7 was required with the river water.

These two series of experiments gave the best results for using yeasts and/or acid-fast organisms as indicator organisms instead of the total coliforms. While the removal of the yeasts and acid-fast organisms generally paralleled that of the total coliforms with alum and ferric chloride as the chemical coagulants, yeast removals were 100 times less than that recorded for the total coliforms in the experiments with tap water plus kaolinite clay and raw wastewater, and between 10-100 times less than that for the total coliforms in the river water experiments with lime. In these two series of experiments (series 6 and 7), total coliform removal ranged from 99.7 to 99.98 percent, yeast removal ranged from 97-99.8 percent, and the removal of acid-fast organisms was estimated to be approximately 99.8 percent (Table 21). A high pH can inactivate microorganisms and, admittedly, the observed results indicating a higher removal of total coliforms than yeasts may be an effect of the high pH in these experiments and a superior resistance of the yeasts to these conditions.

REMOVAL OF INDICATOR ORGANISMS BY SAND FILTRATION

Figure 35 shows the results of a typical sand filtration experiment performed with dechlorinated tap water plus a 1 percent wastewater inoculum as a source of seed organisms. In this experiment, the filter was operated for 20 hr with an applied flow rate of between 167-190 mℓ/min and a mean rate of 180 mℓ/min. This rate gave a loading of 159 ℓ/min/m^2. Yeast removal ranged from 86-97 percent. Removal of acid-fast organisms was initially 65 percent but increased to 76 percent after 4 hr of filtration. After this time, a continuous decrease in removal was noted until the end of the filter run when the removal was only approximately 40 percent. Data for the removal of total coliforms were very similar to that of the acid-fast organisms. Initial removals of total coliforms were nearly 70 percent and showed a gradual reduction to 42 percent removal at the end of 20 hr. It should be noted that the test waters were applied directly to the filter without any prior treatment, i.e., coagulation-sedimentation.

Results of the above experiment, another filter run (12 hr) with dechlorinated tap water plus 1 percent wastewater, and two 36 hr filter runs with pure culture organisms added to dechlorinated tap water are summarized in Table 24. In this table, the removal efficiencies are reported at various times during the filtration period. Data are presented as combined results of the two experiments with wastewater inoculum and the two experiments with pure culture organisms. Removal percentages are recorded as an average for a given length of filter run if the results of the two experiments were within 5 percent of the average, and as a range of values if the results differ by more than 5 percent of the average. For example, the initial removal (0 hr) of total coliforms in the two wastewater inoculum experiments was 55 percent in one experiment and 70 percent in the other. This result is presented as the range 55-70 percent. After 6 hr of filtration, total coliofrm removal in one experiment was 68 percent and 72 percent in the second test. These results are reported in Table 24 as an average 70 percent removal.

It should be pointed out that the rate of filtration in all four experiments was not the same. Loading rates in the two wastewater inoculum experiments were 119 ℓ/min/m^2 and 159 ℓ/min/m^2 while in the two pure culture experiments the rates averaged 106 and 143 ℓ/min/m^2, respectively. Removal results with each test water compared well between the two experiments for each water and are therefore reported as discussed above. Results reported for 18 hr of filtration in the wastewater inoculum experiments represent data from only one experiment since one experiment was terminated after 12 hr. Data presented in Table 24 for the wastewater inoculum experiments indicate a greater removal of yeasts as compared to the removal of acid-fast organisms and total coliforms.

In the two wastewater inoculum tests, the control densities of total coliforms and yeasts were 1×10^5 and 2.2×10^5/100 mℓ, respectively. The control densities of acid-fast organisms ranged from 2.3×10^3/100 mℓ in one experiment to 2.1×10^2/100 mℓ in the other experiment. These two different values with the acid-fast organisms apparently did not affect the removal percentage in the two experiments.

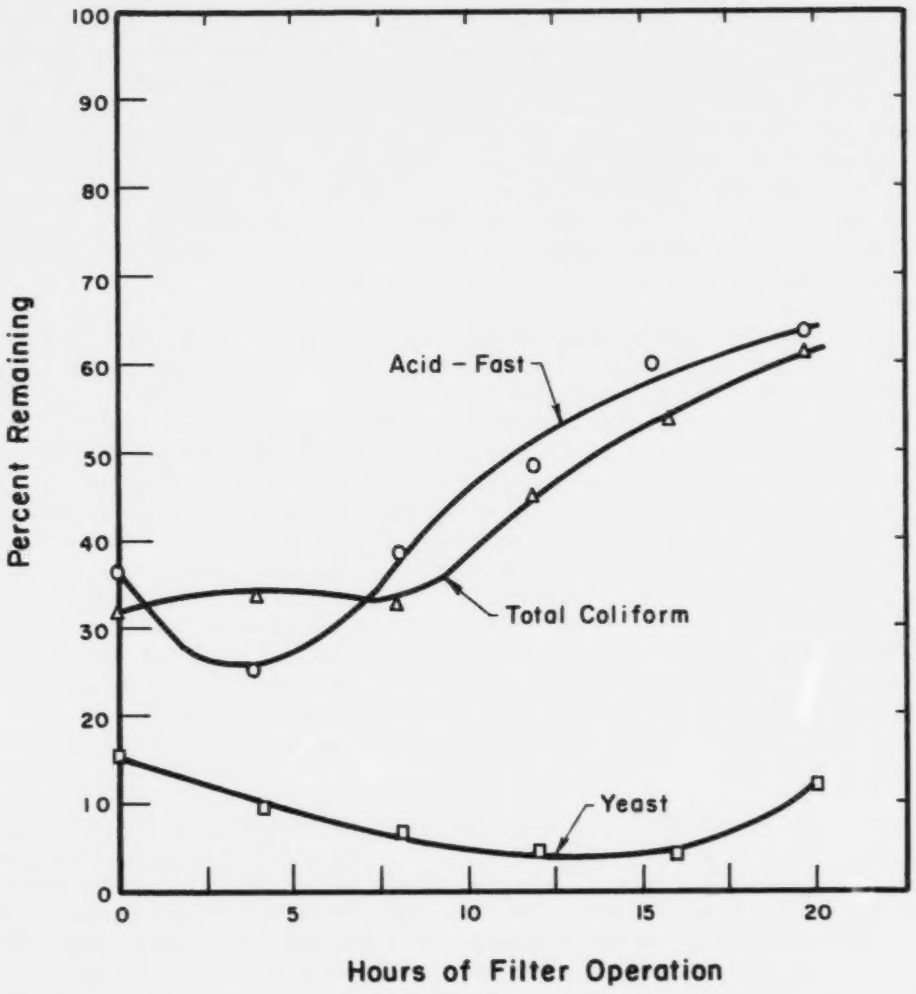


Figure 35. Removal of Indicator Organisms by Sand Filtration Using Tap Water Plus 1.0 Percent Raw Wastewater Inoculum

TABLE 24. REMOVAL OF INDICATOR ORGANISMS BY SAND FILTRATION

	Percent removal at various times during filter run, hr					
	0	6	12	18	24	32
1% Wastewater Inoculum						
Total coliforms	55-70	70	55	45*	--	--
Acid-fast organisms	67	66	43-54	40*	--	--
Yeasts	87	96	97	97*	--	--
Pure Culture Organisms						
E. coli	35-50	23	18-49	16-41	13-51	5-35
M. fortuitum and *M. phlei*	47	53	42	34	18-32	21
C. parapsilosis	62-78	61-76	61-76	60	59	60

*Data from one filter run only

In the pure culture experiments, it was observed that the representative acid-fast organisms, *M. fortuitum* and *M. phlei*, were removed to the same degree as *E. coli*. Data for *E. coli* showed a fairly large range of removal efficiencies between the two experiments. The density of *E. coli* in the feed water in the two experiments was 2.4×10^3 and 5.1×10^4/100 mℓ in the low rate (106 ℓ/min/m^2) and the high rate (143 ℓ/min/m^2) experiments, respectively. The low removal percentage for *E. coli* in Table 24 was observed with the low rate application experiment while the larger percentage removal corresponds to the higher rate experiment. The density of the acid-fast organism (*M. fortuitum* plus *M. phlei*) in the feed water was 3.1×10^3 and 6.2×10^3/100 mℓ in the two experiments. *C. parapsilosis*, the representative yeast, showed the largest removal which was fairly consistent between 60 and 78 percent in both experiments. In the case of *C. parapsilosis*, the control density in the feed water in the two experiments was 1.2×10^3 and 4.2×10^3/100 mℓ in the low rate and high rate experiments, respectively. That *C. parapsilosis* was removed to a higher degree than *E. coli* and the two acid-fast organisms is consistent with the results of the wastewater inoculum experiments. It is conceivable that the larger cell size of the yeast organism, as compared to that of coliforms and the acid-fast organisms, may have contributed to the greater removal of the yeasts.

CONTINUOUS OZONATION STUDIES

Ozonation studies were performed in the three reactors previously described, using pure cultures of C. *parapsilosis*, M. *fortuitum*, E. *coli*, S. *typhimurium*, and poliovirus type 1 (Mahoney). Mixed culture studies using these organisms were also carried out to determine the relative resistance of these organisms to ozone. The following parameters which influence the efficacy of ozone disinfection were studied:

a) Ozone residual and ozone bubbles
b) Ozone residual only
c) Ozone bubbles only
d) Effect of mixing in the reactor
e) Initial density of organisms
f) Effect of pH
g) Effect of temperature
h) Presence of UV light

Pure Culture Studies

Effect of Ozone Residual and Ozone Bubbles --

Reactor arrangement No. 1 was employed to study the effect of ozone residual plus ozone bubbles. Mixing was provided by a gaseous mixture of ozone and air, supplied at the bottom of the reactor, and the ozone residual was monitored at each detention time (DT). Figures 36 and 37 show the survival of C. *parapsilosis* and M. *fortuitum*, respectively, for a detention time of 24 sec.

Effect of Ozone Residual --

Using experimental arrangement No. 3, the effect of ozone residual without bubbles on the inactivation of C. *parapsilosis* and M. *fortuitum* was determined. Figures 36 and 37 show the percent survival of these organisms vs. ozone residual for a 24 sec detention time.

Comparison of Figures 36 and 37 clearly show that inactivation of both test organisms was greater with reactor arrangement No. 1 than with arrangement No. 3 for a given ozone residual. This indicates that the presence of ozone bubbles, along with ozone residual, was more effective in inactivating the test organisms than ozone residual alone.

A conceptual model was prepared to explain this phenomenon. Based on the film theory of gas transfer, when an organism comes in contact with ozone in aqueous solution, a liquid film develops around the aqueous layer associated with the cell wall of the organism. This adsorbed aqueous layer may be considered an integral part of the cell wall of the organism. Also, according to the film theory, a concentration gradient similar to the gas-liquid interface develops in the liquid film surrounding the aqueous layer. Generally, the thickness of the liquid film at the gas-liquid interface is much larger than the diameter of the organism. Further, microorganisms tend to concentrate at the gas-liquid interface. Because of their presence in the interface, microorganisms are surrounded by ozone concentrations which are higher than the bulk liquid ozone concentration. Therefore, with the same ozone residual in the bulk liquid the tendency for inactivation would be greater for a cell which is either in direct contact with an ozone bubble or

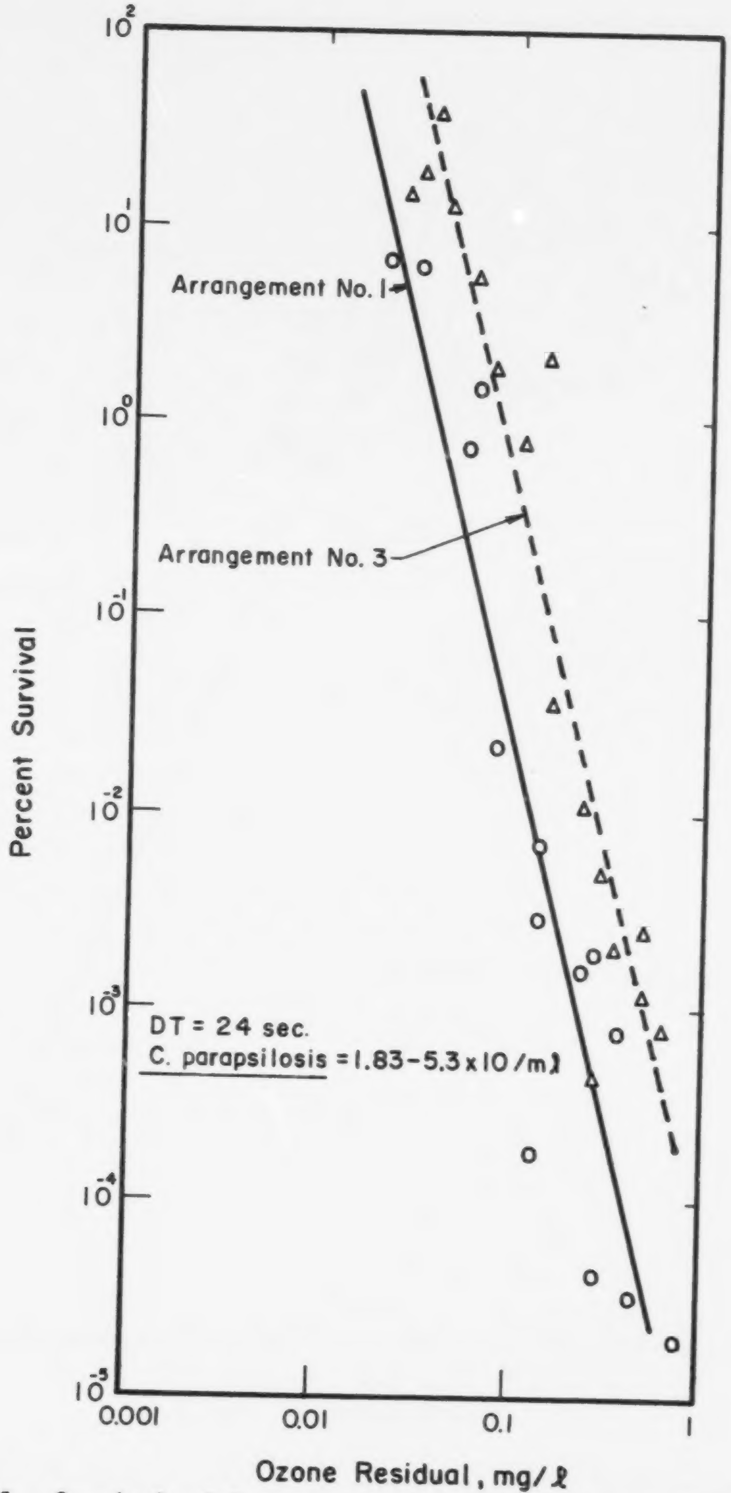


Figure 36. Survival of C. *parapsilosis* in Reactor Arrangement Nos. 1 and 3

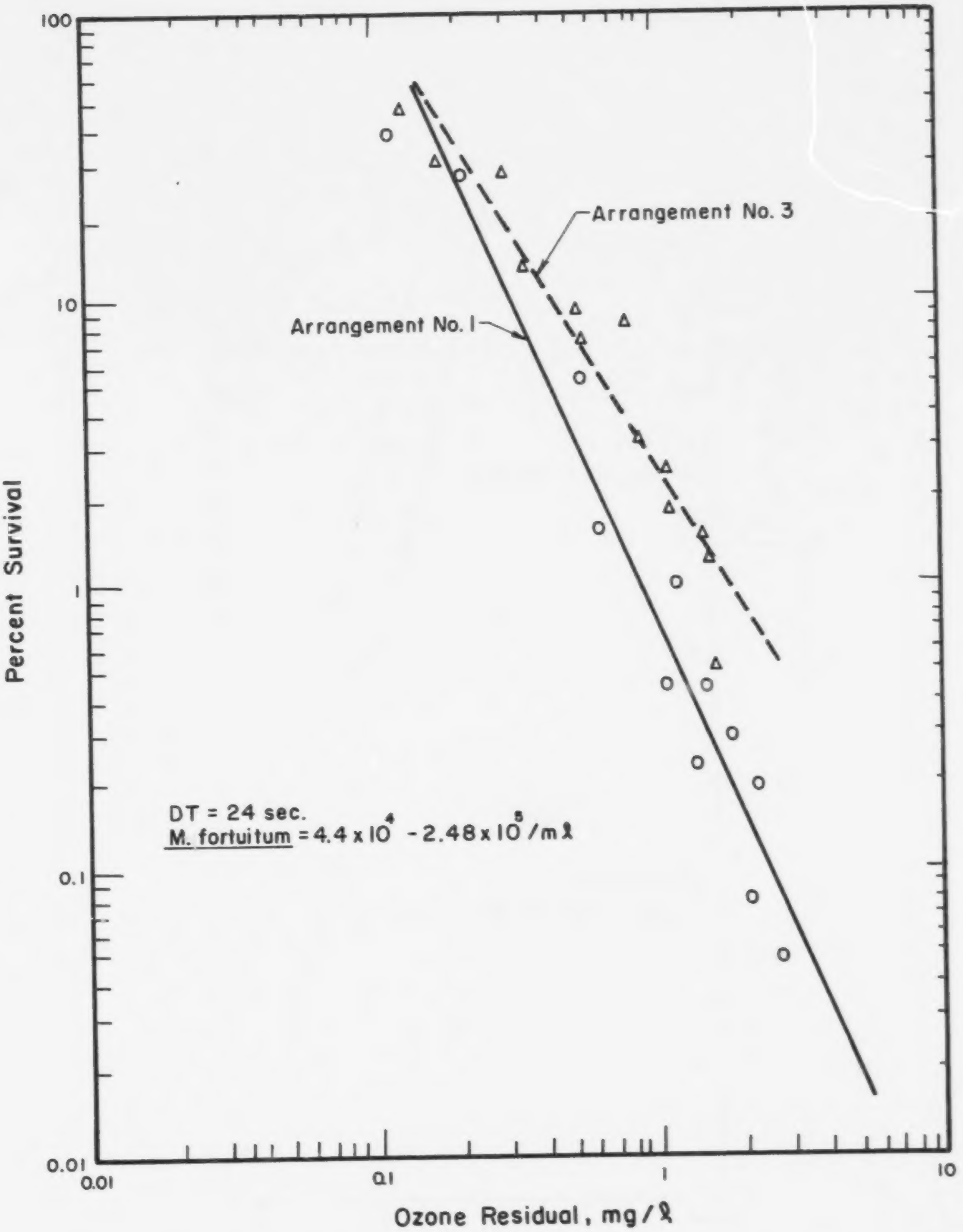


Figure 37. Survival of M. *fortuitum* in Reactor Arrangement Nos. 1 and 3

within the liquid film than for a cell in the bulk liquid. This concept of concentration effect is in agreement with the observed inactivation data as shown in Figures 36 and 37, i.e., greater inactivation in a system having both ozone bubbles and a residual.

Effect of Ozone Bubbles --

Studies involving inactivation by ozone bubbles in the absence of any residual were performed in reactor arrangement No. 2, where the ozone residual was destroyed by the addition of an appropriate quantity of sodium thiosulfate. Survival curves for M. *fortuitum* and C. *parapsilosis* are presented in Figures 38 and 39, respectively; the data indicate a slight amount of inactivation even in the absence of an ozone residual. This may be due to the fact that an ozone residual may have existed for a short period of time before being reduced by sodium thiosulfate. No information is available about the reaction kinetics of ozone and thiosulfate except that it is believed to be an extremely rapid reaction.

Effect of Mixing --

Ozone decomposition rate has been reported by Hewes *et al.* (15) to be proportional to the rate of mixing. Thus, higher mixing rates result in lower ozone residuals and hence lower inactivation. The effect of mixing on the ozone decomposition rate and the subsequent inactivation of the test organisms was studied in reactor arrangement No. 3. The results are presented in Table 25. It is apparent that a higher decomposition rate of ozone and, thus less inactivation of both organisms, occurred as the rate of agitation increased.

Effect of pH --

Stumm (16) reported that the rate of ozone decomposition was greater with higher pH values in aqueous solution; this effect was associated with the catalytic activity of the hydroxyl ion, as proposed by Hewes and Davidson (17). Therefore, a change in pH results in a different ozone residual at a constant ozone gas flow rate. To study this effect, the ozone/air gas flow rate was maintained at 0.5 ℓ/min with a gaseous ozone concentration of 16.8 mg/ℓ in reactor arrangement No. 1. Phosphate buffer was used to obtain pH 5.5 and 7, and borate buffer was used for pH 8.5 and 10. Organism survival curves for these experiments are presented in Figures 40 and 41, from which it can be seen that the percent survival is higher at higher pH values with a constant ozone gas flow rate. Since the results of the pH studies given above do not reflect the sole effect of pH on inactivation, experiments were designed to maintain the residual ozone at a constant level while varying the pH. Survival curves for this set of experiments are presented in Figure 42, which indicates that the percent survivals at different pH values are approximately the same for similar ozone residuals.

Effect of Temperature --

A change in temperature is known to affect the rate of inactivation of microorganisms by a disinfectant, presumably because of the resulting change in diffusion rate and in enzymatic reaction rate. To study this effect, experiments were performed in reactor arrangement No. 1, using a procedure similar to that employed in studying the effect of pH.

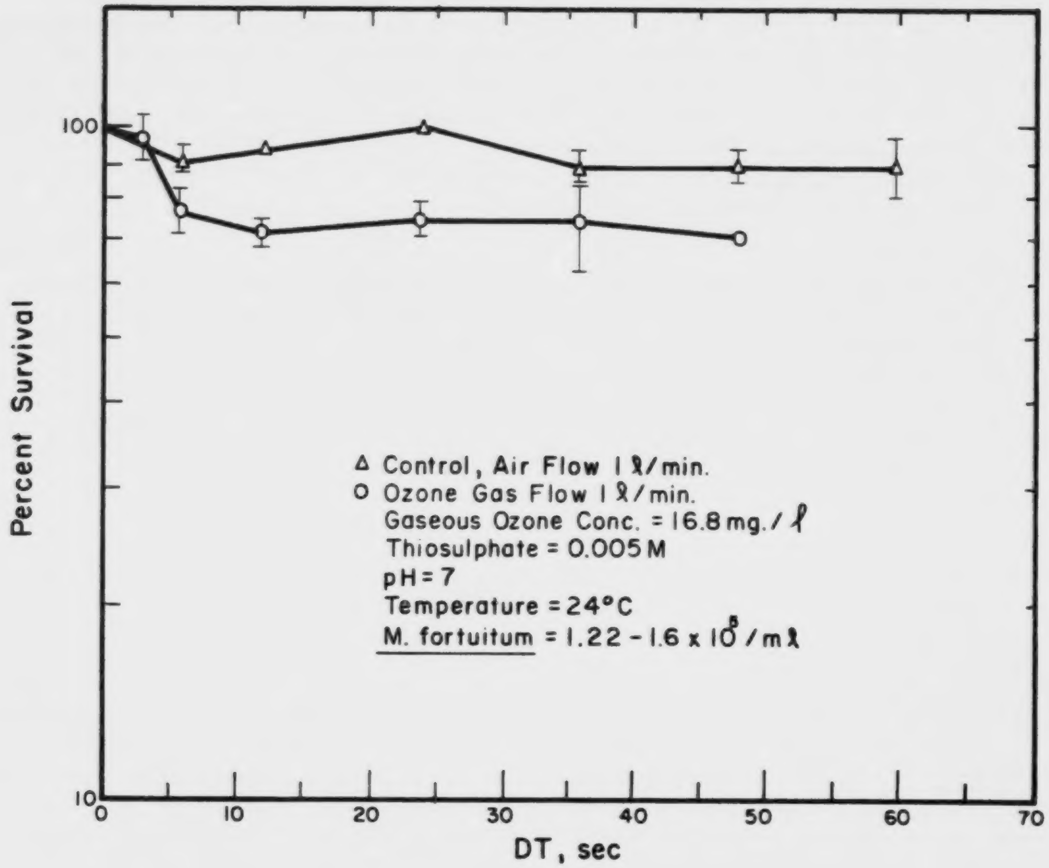


Figure 38. Survival of M. *fortuitum* in the Presence of Ozone Bubbles with no Ozone Residual, Reactor Arrangement No. 2

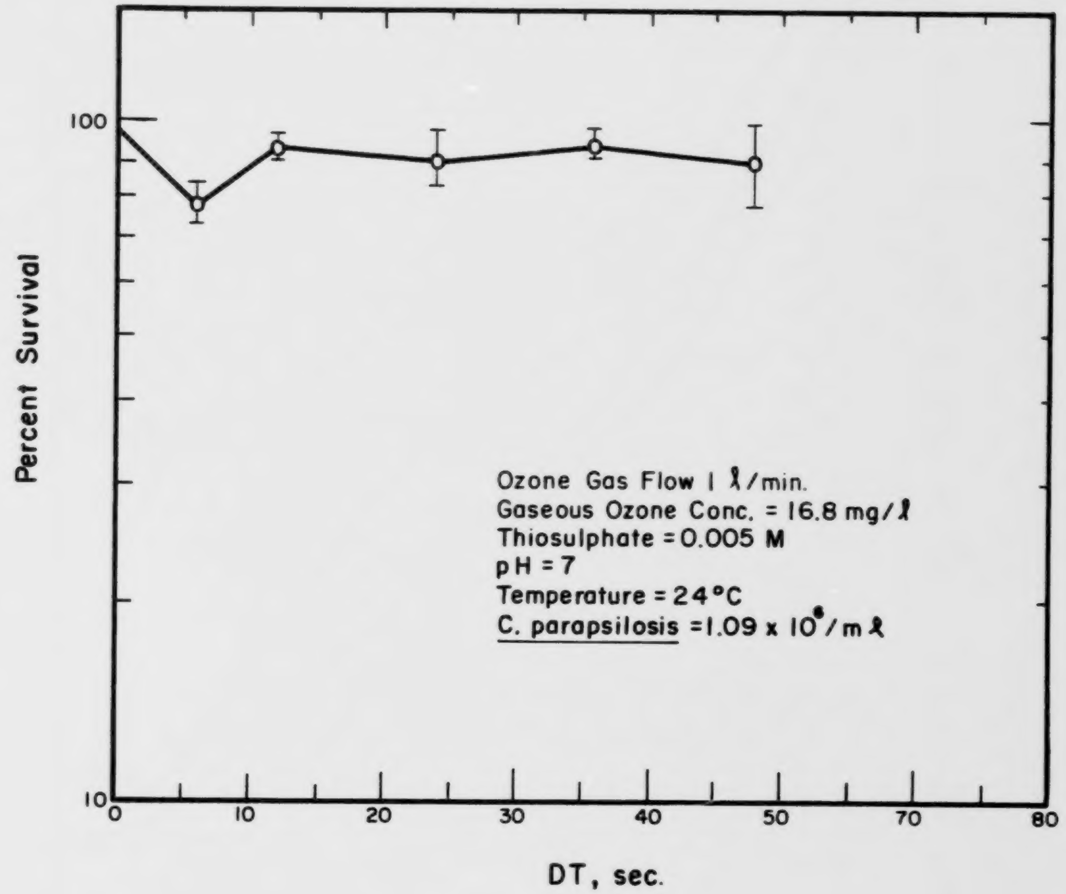


Figure 39. Survival of C. *parapsilosis* in the Presence of Ozone Bubbles with no Ozone Residual, Reactor Arrangement No. 2

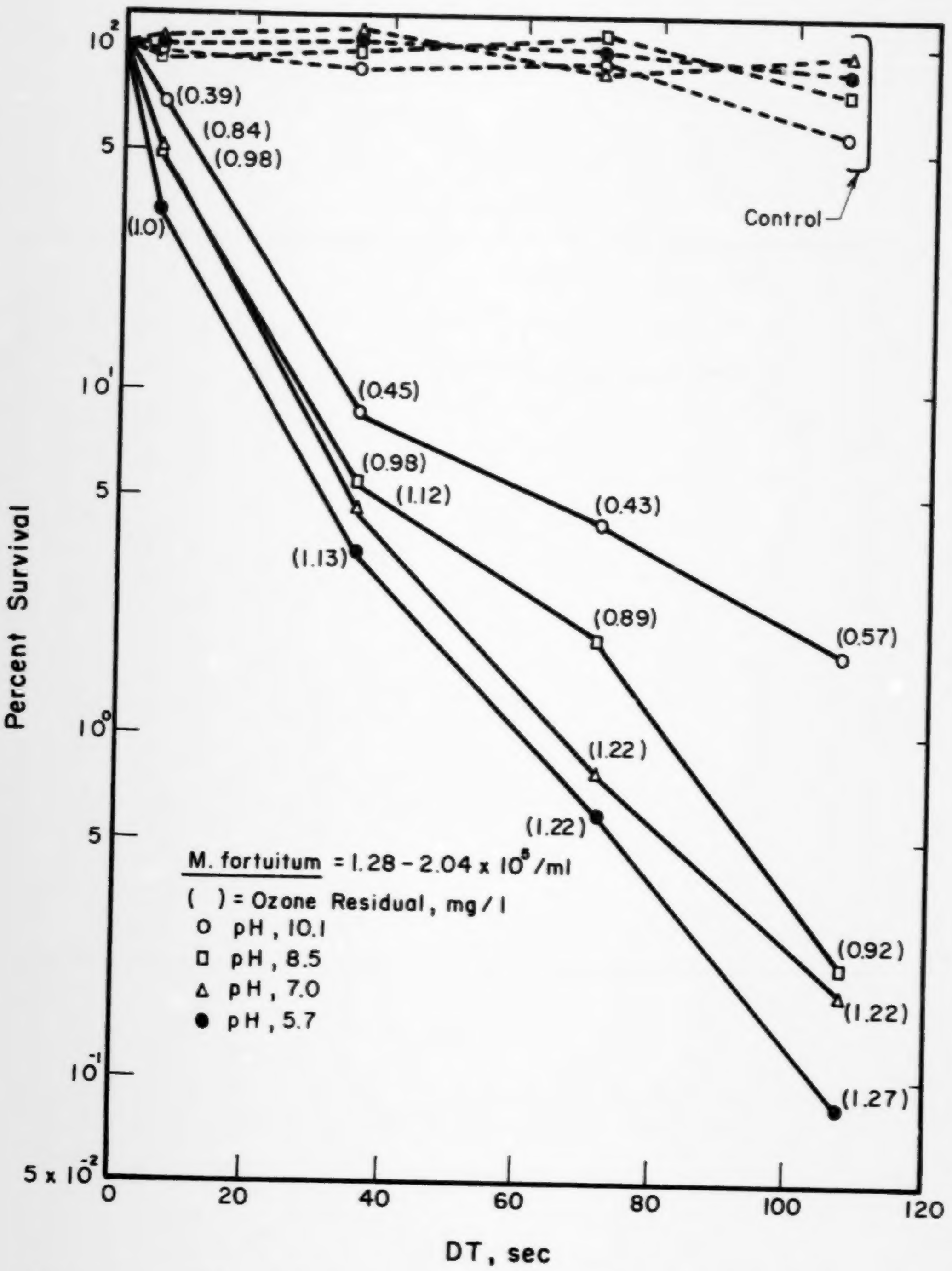


Figure 40. Effect of pH on the Survival of M. *fortuitum* at a Constant Rate of Applied Ozone

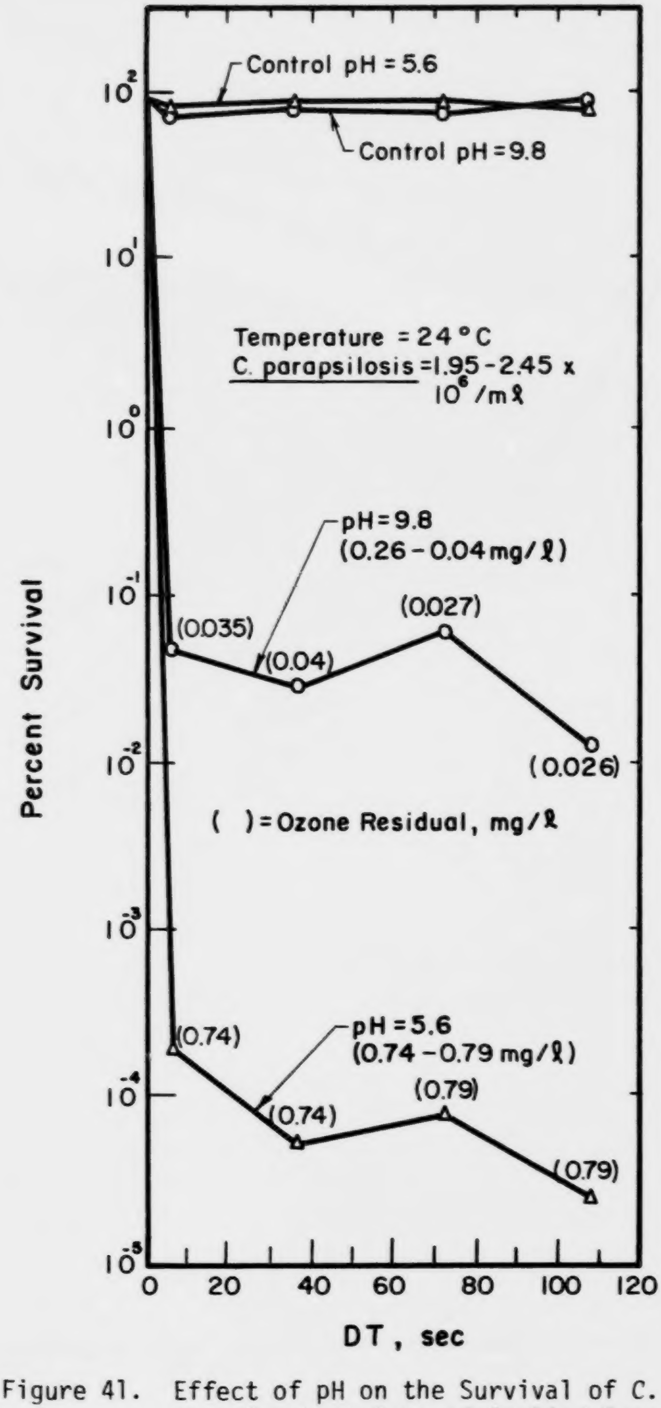


Figure 41. Effect of pH on the Survival of C. *parapsilosis* at a Constant Rate of Applied Ozone

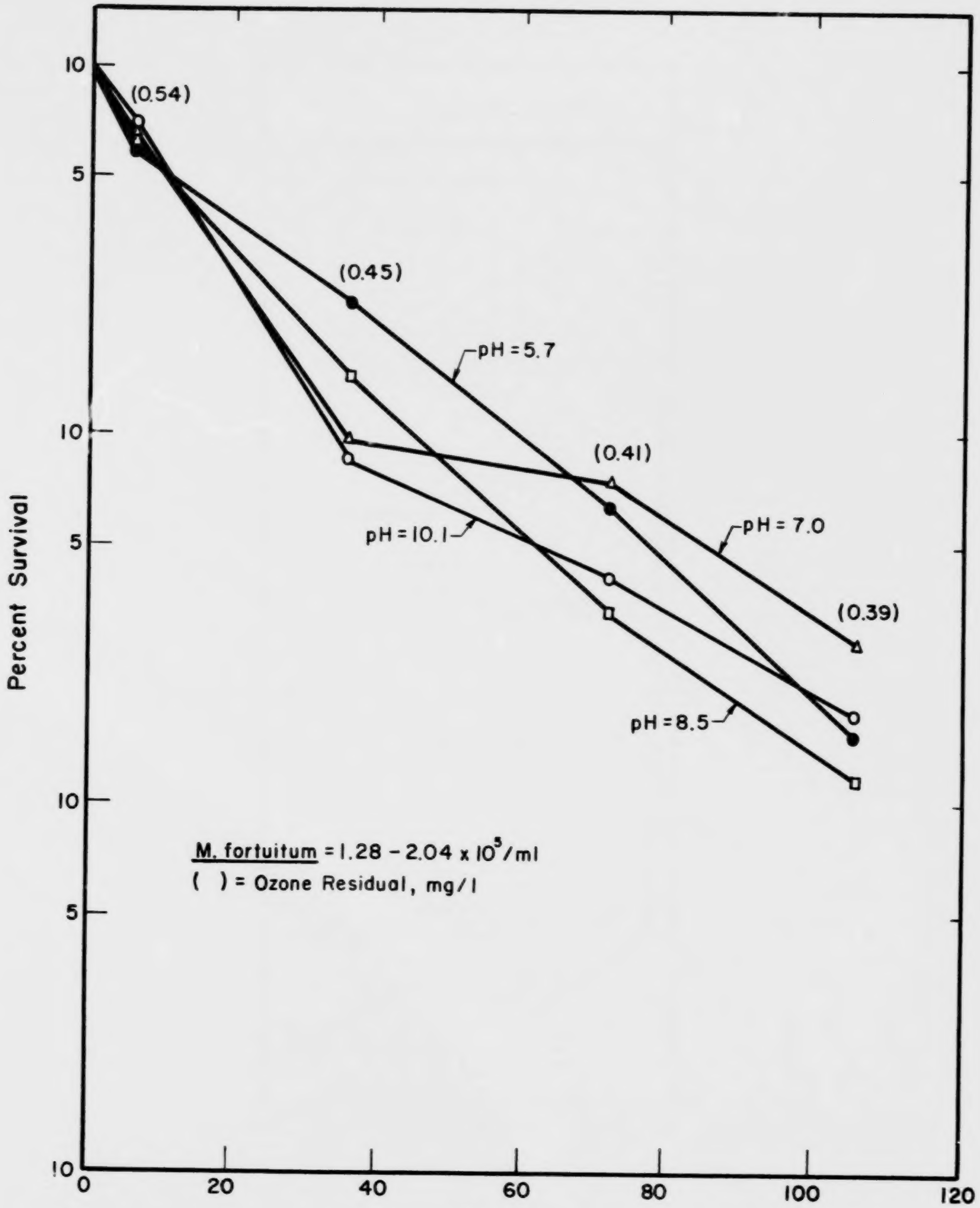


Figure 42. Effect of pH on the Survival of M. *fortuitum* for a Constant Ozone Residual at a Given DT

TABLE 25. EFFECT OF MIXING RATE ON THE SURVIVAL OF M. *fortuitum* AND C. *parapsilosis* IN EXPERIMENTAL ARRANGEMENT NO. 3 FOR A DT OF 24 SECONDS

Applied ozone mg/ℓ	Mixing rpm	Ozone residual mg/ℓ	Number cells/ mℓ	Percent survival
			M. *fortuitum* Control, 2.32×10^5	100.00
1.27	63	0.58	2.19×10^4	9.44
1.27	210	0.55	2.19×10^4	12.54
1.24	345	0.52	3.23×10^4	13.91
			C. *parapsilosis* Control, 3.1×10^6	100.00
1.12	63	0.257	4.65	0.00015
1.15	210	0.245	24.20	0.00078
1.15	345	0.233	957.00	0.0308

In the first set of experiments, the applied ozone level was maintained constant at 15.8 mg/ℓ, with an ozone/air gas flow rate of 0.5 ℓ/min. The reactor temperatures studied were 9°, 20°, 30°, and 40°C. Figure 43 shows the results of the experiments using M. *fortuitum* as the test organism. An increase in temperature resulted in a higher degree of inactivation of M. *fortuitum* even though the ozone residual was considerably lower at the higher temperatures. A control experiment was also performed at 30° and 37°C in the absence of ozone but with an air flow rate of 0.5 ℓ/min. Results in Figure 43 indicate that a small degree of inactivation occurred presumably due to the direct effect of a temperature increase on inactivation.

Since the ozone residual was observed to be one of the most important parameters affecting ozone disinfection, the ozone residual must be held constant in order to study the sole effect of temperature on inactivation. Therefore, another set of experiments was performed at a constant ozone residual at a given detention time for four different temperatures, i.e., 9°, 20°, 30°, and 37°C. The survival data for M. *fortuitum* in Figure 44 clearly indicate the true effect of temperature on inactivation, i.e., the degree of inactivation increases significantly with an increase in temperature and at a constant ozone residual.

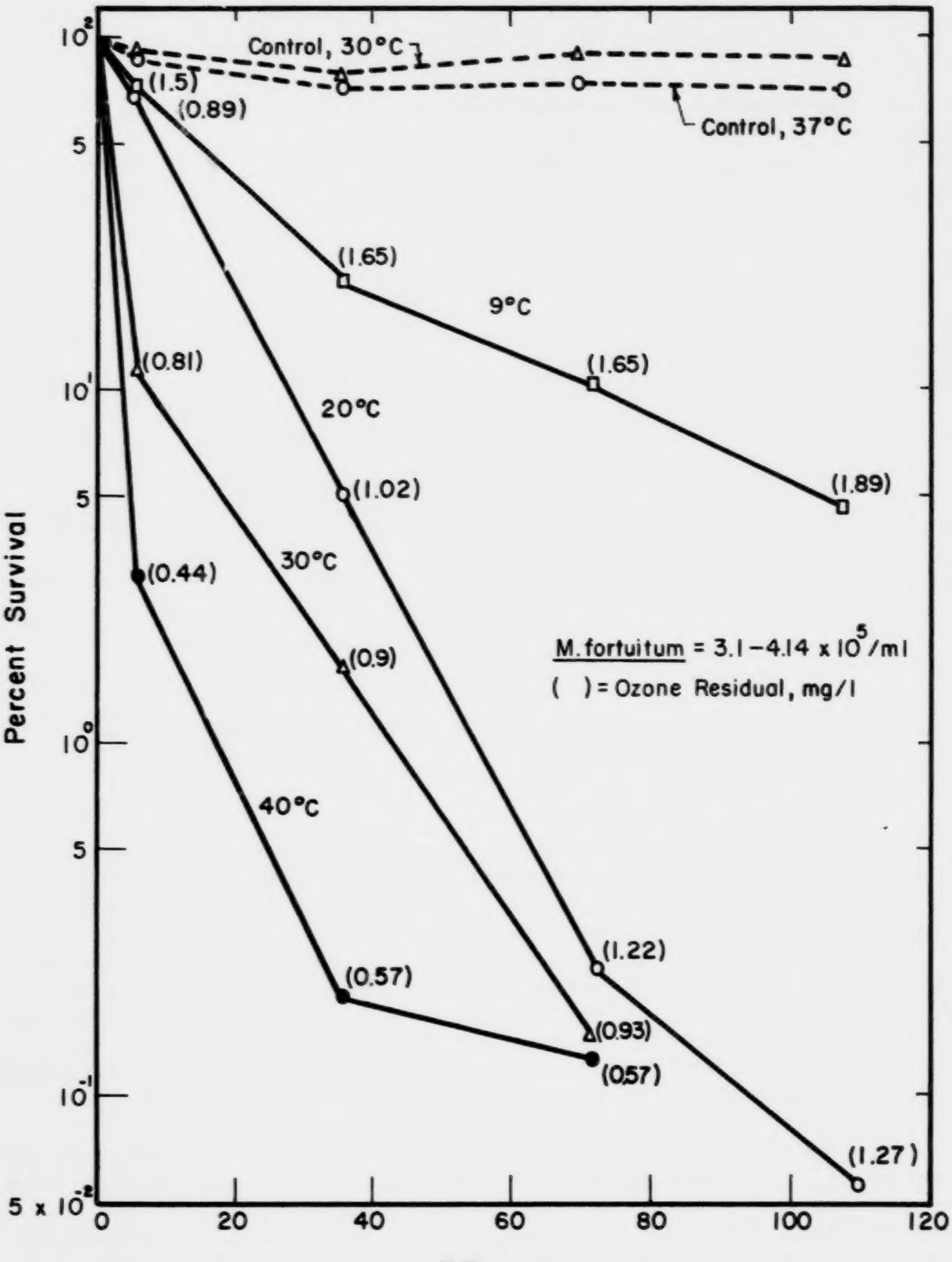


Figure 43. Effect of Temperature on the Survival of M. *fortuitum* at a Constant Rate of Applied Ozone

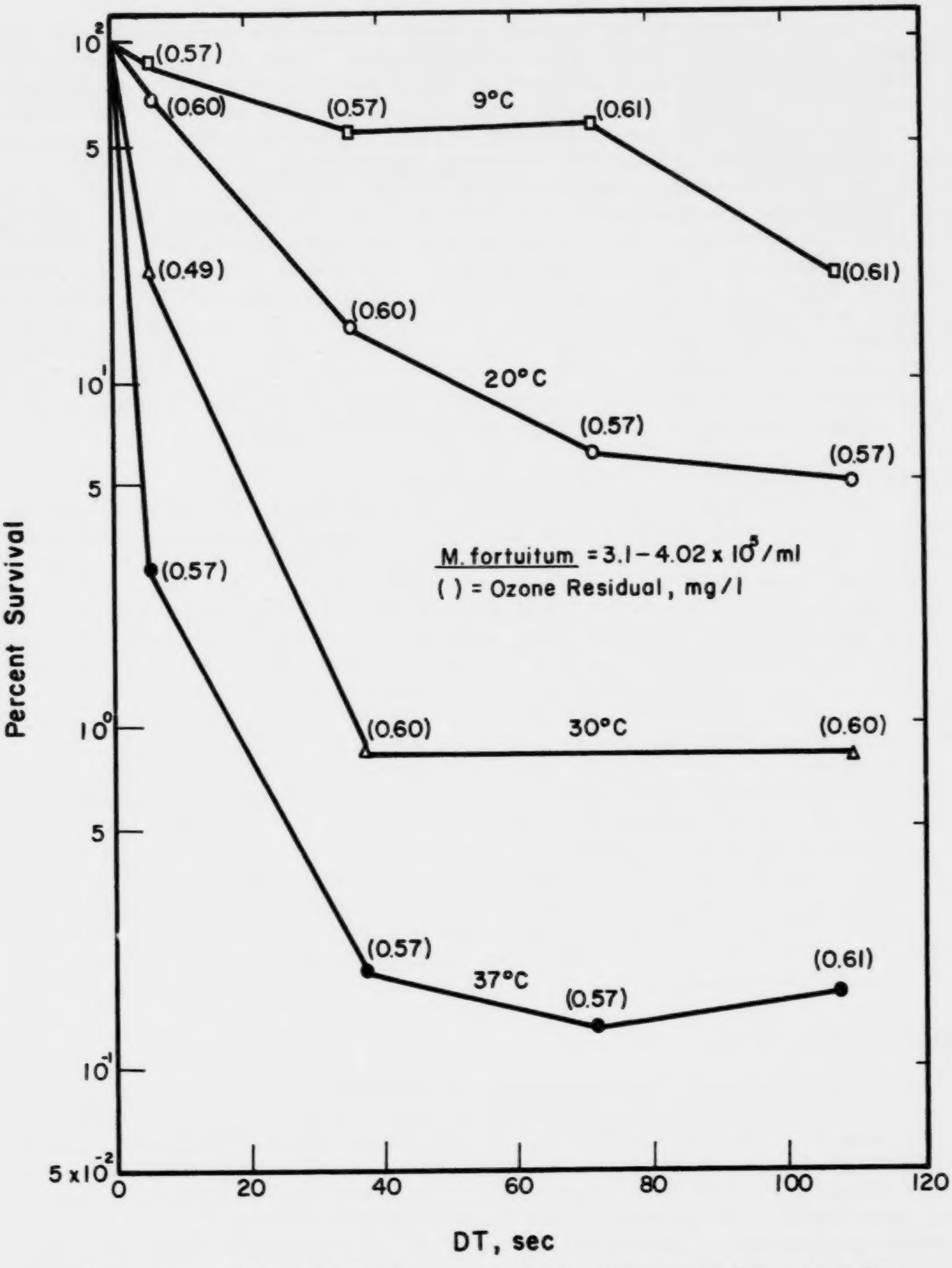


Figure 44. Effect of Temperature on the Survival of M. *fortuitum* for a Constant Ozone Residual at a Given DT

Effect of Ultraviolet (UV) Light --

A study was undertaken to investigate whether the presence of UV light augments inactivation of M. *fortuitum* by ozone. This study was conducted in a fermentor (New Brunswick Scientific Co., New Brunswick, NJ) consisting of a 15 cm dia and 30 cm long Pyrex glass reactor having an effective volume of 4 ℓ. This reactor was equipped with a 15 w low-pressure mercury germicidal lamp with the major ultraviolet energy being emitted at a wavelength of 253.7 nm. The gaseous ozone concentration was 16.8 mg/ℓ. Survival curves from this study are presented in Figure 45. It may be noted that comparable inactivation of M. *fortuitum* occurred with the three different conditions, i.e., UV light, UV light plus ozone, and ozone alone. However, the importance of ozone residual in the presence of UV light becomes less significant because the degree of inactivation of M. *fortuitum* is increased in spite of a decrease in the ozone residual. It can also be seen in Figure 45 that UV light alone is a strong disinfecting agent. Therefore, the increase in inactivation for UV light plus ozone may be due to the application of UV light rather than to the catalytic effect of UV light.

Effect of Initial Density of Microorganisms --

Preliminary experiments indicated that the degree of inactivation by ozone was profoundly affected by the initial organism density. Therefore, three different initial densities of C. *parapsilosis* were studied with respect to inactivation in reactor arrangement No. 3. An applied dissolved ozone concentration was maintained at 0.15 mg/ℓ for the three initial yeast densities. Survival data for C. *parapsilosis* are given in Figure 46, which indicate that the percent survival is greater for higher initial organism density. It may be noted that a 4 log reduction occurred when the initial density of yeast was 1.35 x 10^5 cells/mℓ, while little observable inactivation took place when the initial density was 1.55 x 10^7 cells/mℓ. The limited degree of inactivation seen in Figure 46 for the two highest organism densities can be attributed to the substantial ozone demand exerted by the large amount of organic cell matter present. The measured TOC for the system having an initial organism density of 1.55 x 10^7 cells/mℓ was about 120 mg/ℓ, compared to a TOC of approximately 4 mg/ℓ when the initial density of C. *parapsilosis* was 1.35 x 10^5 cells/mℓ. These experiments indicate that 0.15 mg/ℓ of applied dissolved ozone was not sufficient to inactivate yeasts when the initial density of cells was high, such as in the case with 1.55 x 10^7 cells/mℓ.

Mixed Culture Study

Relative Resistance of Microorganisms to Ozonation --

A mixed culture study, including E. *coli*, M. *fortuitum*, C. *parapsilosis*, S. *typhimurium* and poliovirus type 1 (Mahoney), was performed in reactor arrangement No. 1. All of these organisms were grown separately and then added together in the feed reservoir about 15 min before the initiation of the experiment. The reservoir was mixed continuously to provide a homogeneous suspension. The ozone/air gas flow rate was maintained at a rate of 0.5 ℓ/min. The pH and temperature were 7 and 24°C, respectively. It can be seen from Figure 47 that M. *fortuitum* was the most resistant organism of the five, while S. *typhimurium* was the least resistant. E. *coli*,

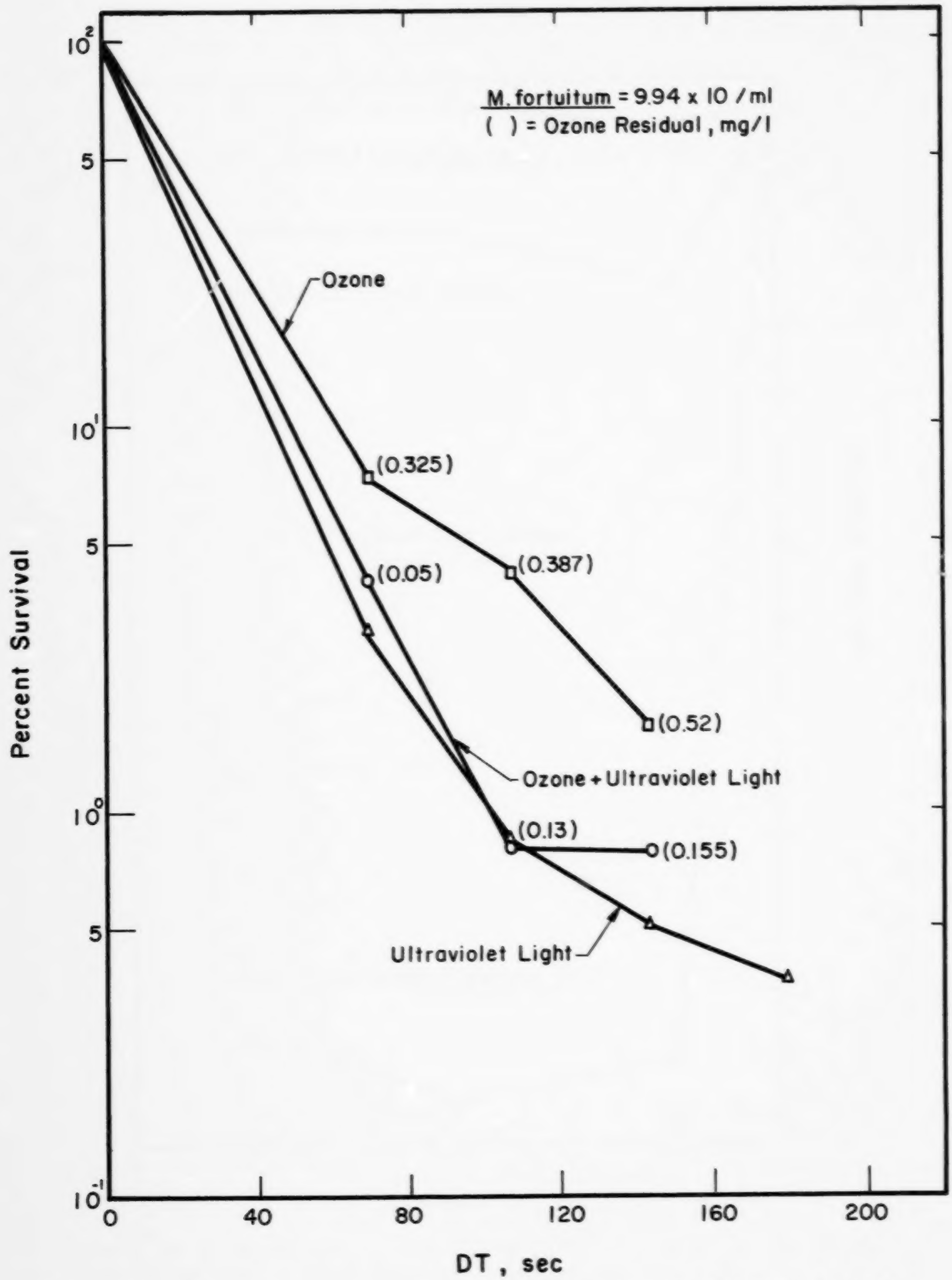


Figure 45. Effect of Ultraviolet Light on the Survival of M. *fortuitum* at a Constant Rate of Applied Ozone

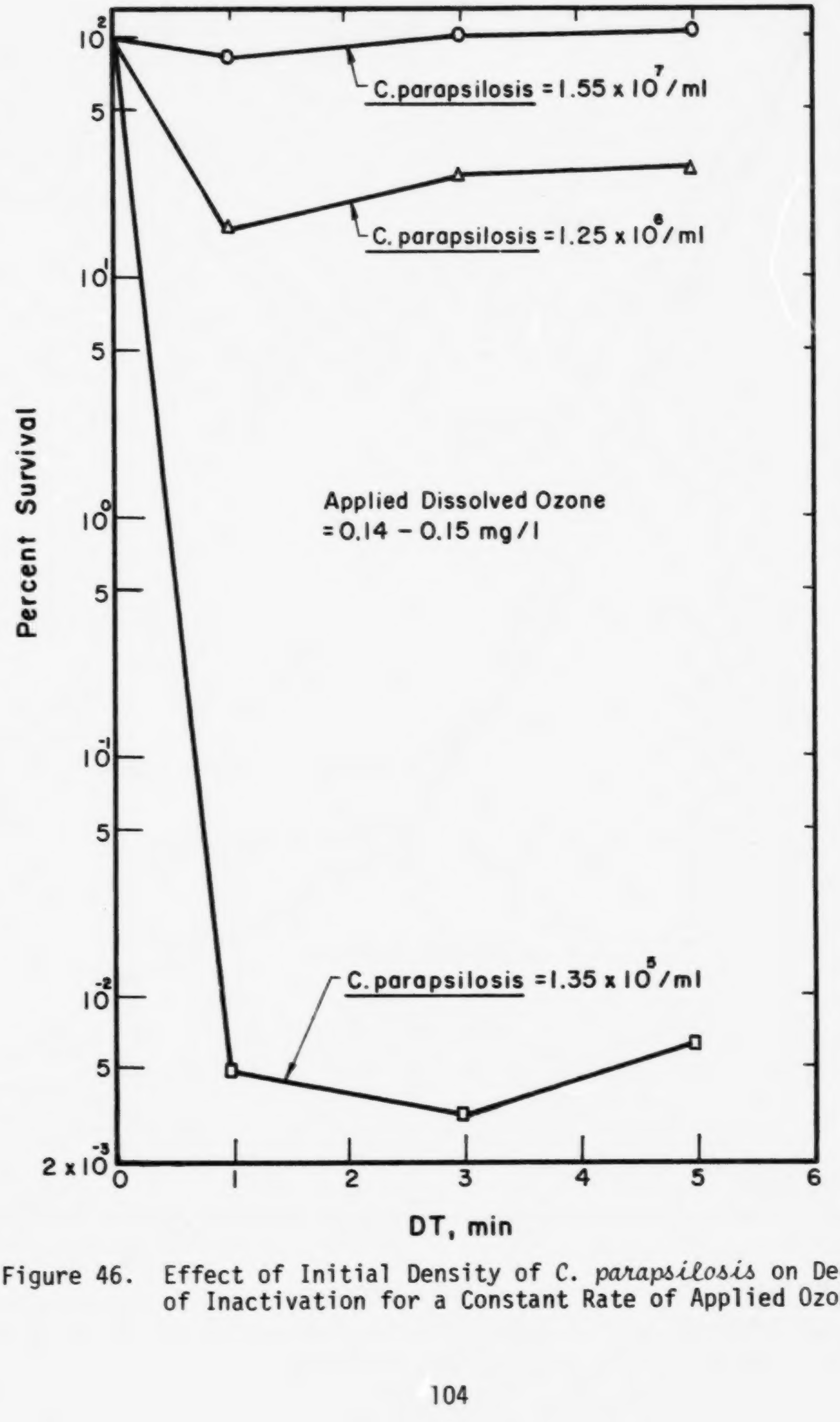


Figure 46. Effect of Initial Density of C. *parapsilosis* on Degree of Inactivation for a Constant Rate of Applied Ozone

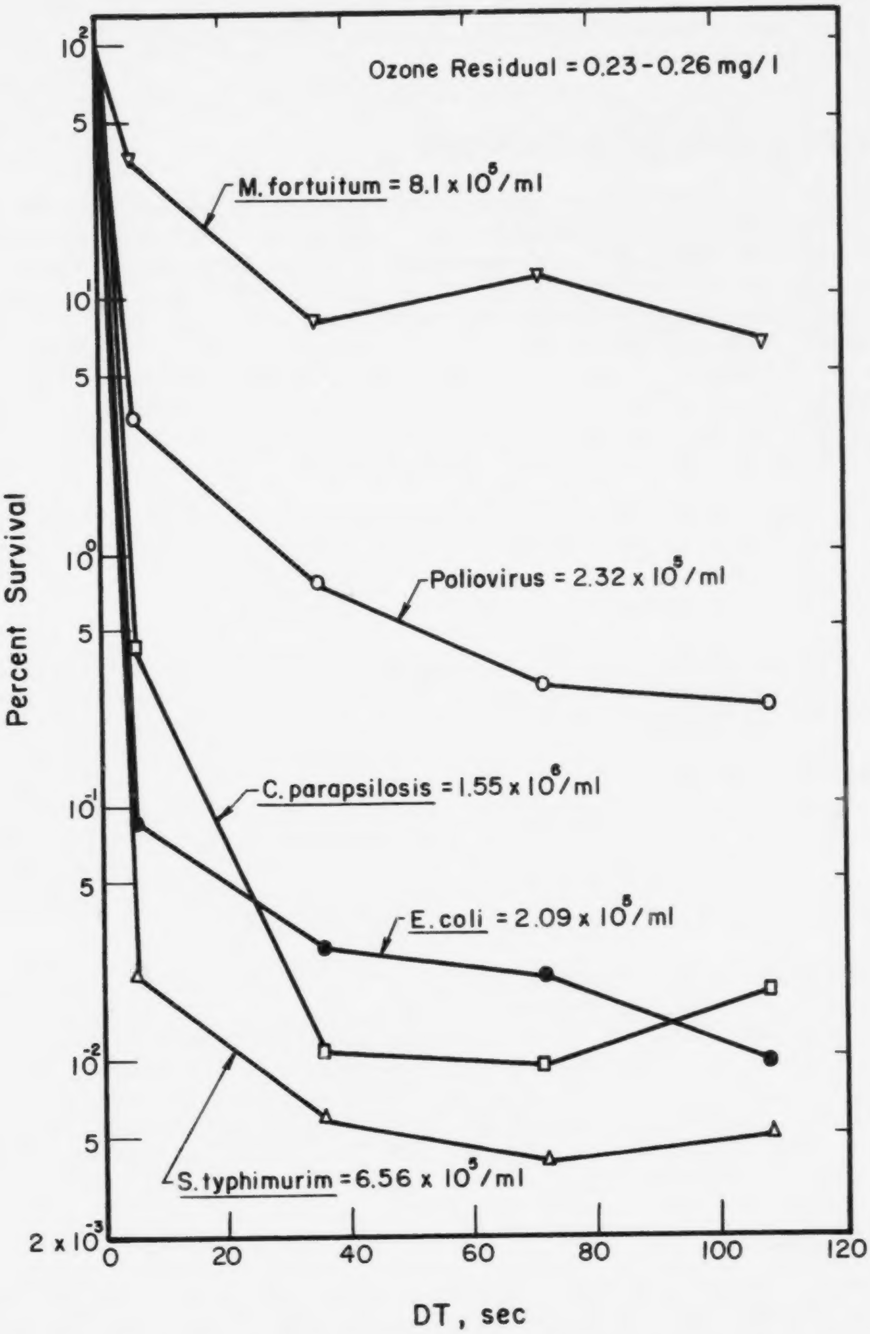


Figure 47. Response of Five Test Organisms to Ozone in a DI Phosphate Buffer Solution

C. *parapsilosis*, and poliovirus showed an increasing order of resistance. The resistance of S. *typhimurium* was comparable to that of E. *coli*; however, poliovirus was more resistant than E. *coli* and C. *parapsilosis* but less resistant than M. *fortuitum*.

MECHANISM OF INACTIVATION BY CHLORINE

If either acid-fast or yeast organisms are to be accepted as a new indicator of disinfection efficiency, it is necessary that they be more resistant to disinfection under all circumstances likely to be encountered. This ability can be ascertained either by extensive field testing or by showing that the increased resistance they exhibit is a logical result of known aspects of their microbial physiology. Since chlorine is currently the most common disinfectant of water and wastewater in the United States, it was decided to investigate the mechanism of resistance of the acid-fast and yeast organisms to free available chlorine, using C. *parapsilosis* and M. *fortuitum* as model organisms, respectively

The question of mechanisms of inactivation by free available chlorine may be considered in two parts: 1) What is the basis for the increased efficiency of HOCl vs. OCl^-?, 2) What is(are) the site(s) of inactivation of free available chlorine within the cell?

The most prevalent hypothesis by way of answering the first question is that the uncharged HOCl molecule is better able to penetrate the cell wall and be taken up by the microorganism (18). Although this theory has been directly confirmed in the case of amoebic cysts (18), bacterial spores (19), and bacteriophage (20), no direct verification has been reported using vegetative bacteria or yeasts, although the hypothesis has been supported, in the case of E. *coli*, by work using organic chloramines (21). In an apparent contradiction to the permeability hypothesis, it has been reported that bacteria take up more chlorine after exposure to free available chlorine at high pH than at low pH (22,23).

In regard to the second question, the classical hypothesis has been one of damage to respiratory enzymes (24,25); however, this hypothesis was developed without benefit of the current level of knowledge of cellular biochemistry (26). More recent work has suggested that damage to the cell permeability barriers may be a cause of inactivation (27,28). In addition, a variety of studies have suggested that chlorine and chloramines can damage cellular deoxyribonucleic acid (DNA) (29-36). Thus, damage to DNA may be at least partially responsible for cell inactivation (29,31).

Chlorine Dynamics

Initial experiments were performed with radioactive chlorine to determine the kinetics and extent of chlorine binding. These studies showed that the association of chlorine with the cells was fairly rapid (Figure 48). It was determined that a contact time of 6 min for E. *coli*, and 30 min for either C. *parapsilosis* or M. *fortuitum* was sufficient to ensure completion of chlorine uptake.

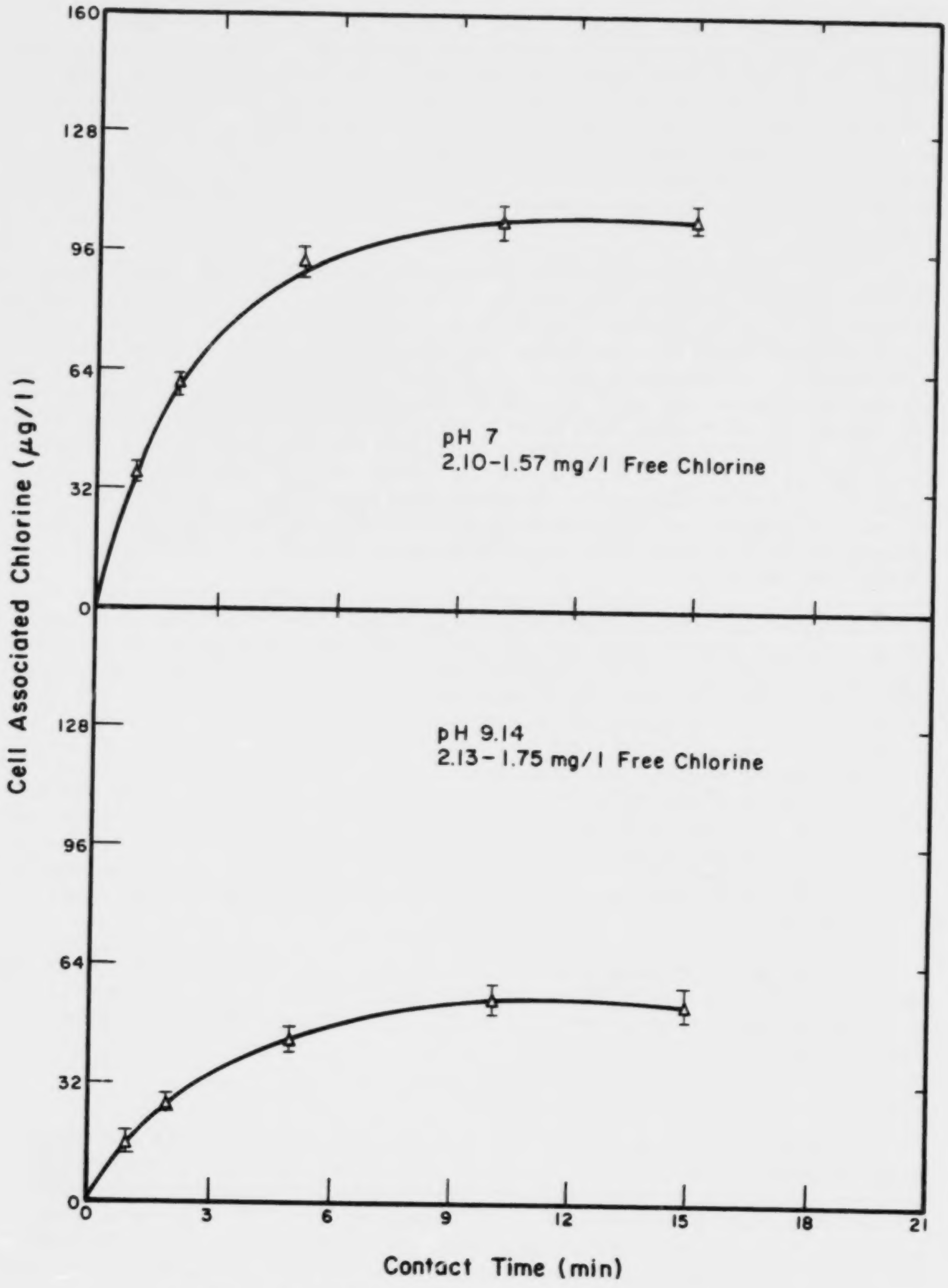


Figure 48. Chlorine Uptake Kinetics of E. *coli*

Using these fixed contact times, further studies on the extent of chlorine interactions with the cells were performed. In these studies, the amount of chlorine associated with the cells was determined using radioactivity measurements, and the initial and final free available chlorine concentrations in solution were measured amperometrically; from a mass balance, it was determined that a significant fraction of the initial free available chlorine was not present at the termination of the experiment as either cell associated chlorine or free residual. This fraction was designated as "reacted chlorine."

It was found that cell-associated chlorine could be adequately described by a Freundlich isotherm (Figure 49). In most cases the amount of chlorine present in association with the cells was much less than the reacted chlorine; in some cases this deviation was more than an order of magnitude. The ability of C. *parapsilosis* and M. *fortuitum* to take up more chlorine at low pH than at high pH was verification of the permeability theory. Further, the amount of cell-associated chlorine was plotted against the theoretical concentration of HOCl (Figure 50). Isotherms for the yeast and acid-fast organisms at pH 7 and 9.14, obtained by linear regressional analysis, were found to be not significantly different from each other. In the case of E. *coli*, a substantial difference in cell-associated chlorine was noted between cells contacted with a constant concentration of hypochlorous acid at pH 7 vs. pH 9.14.

An additional observation from these studies was that there appeared to be a correlation between the amount of chlorine taken up by each organism and its inherent sensitivity to chlorine. On the basis of the continuous flow chlorination studies and similar work performed earlier (2), the order of sensitivity of these organisms towards free chlorine was observed to be E. *coli* > C. *parapsilosis* > M. *fortuitum*. Considering cell size, C. *parapsilosis* has about 20-100 times the surface area and volume of E. *coli*, while M. *fortuitum* is of a similar size to E. *coli*. Based on Figures 49 and 50, after correction for cell size, the order of ease of uptake of chlorine was E. *coli* > C. *parapsilosis* > M. *fortuitum*. The only exception to this relationship was E. *coli* at pH 7 which, it will be shown, may have been due to underestimation of cell-associated chlorine and excessive leakage after chlorination.

On the basis of these experiments, it was determined that the basis for increased efficiency of HOCl as a disinfectant compared to OCl^- was its enhanced uptake by microorganisms due to its increased ability to penetrate the cell wall.

Biochemical Effects

To determine the nature of the lethal event by which chlorine inactivates microorganisms, it was necessary to perform experiments monitoring various aspects of cellular activity presumably influenced by chlorination. In the first set of experiments, cells suspended in CDFB were dosed with chlorine, held for a given contact time, and dechlorinated with thiosulfate. Following this, the test organism was inoculated into an appropriate liquid

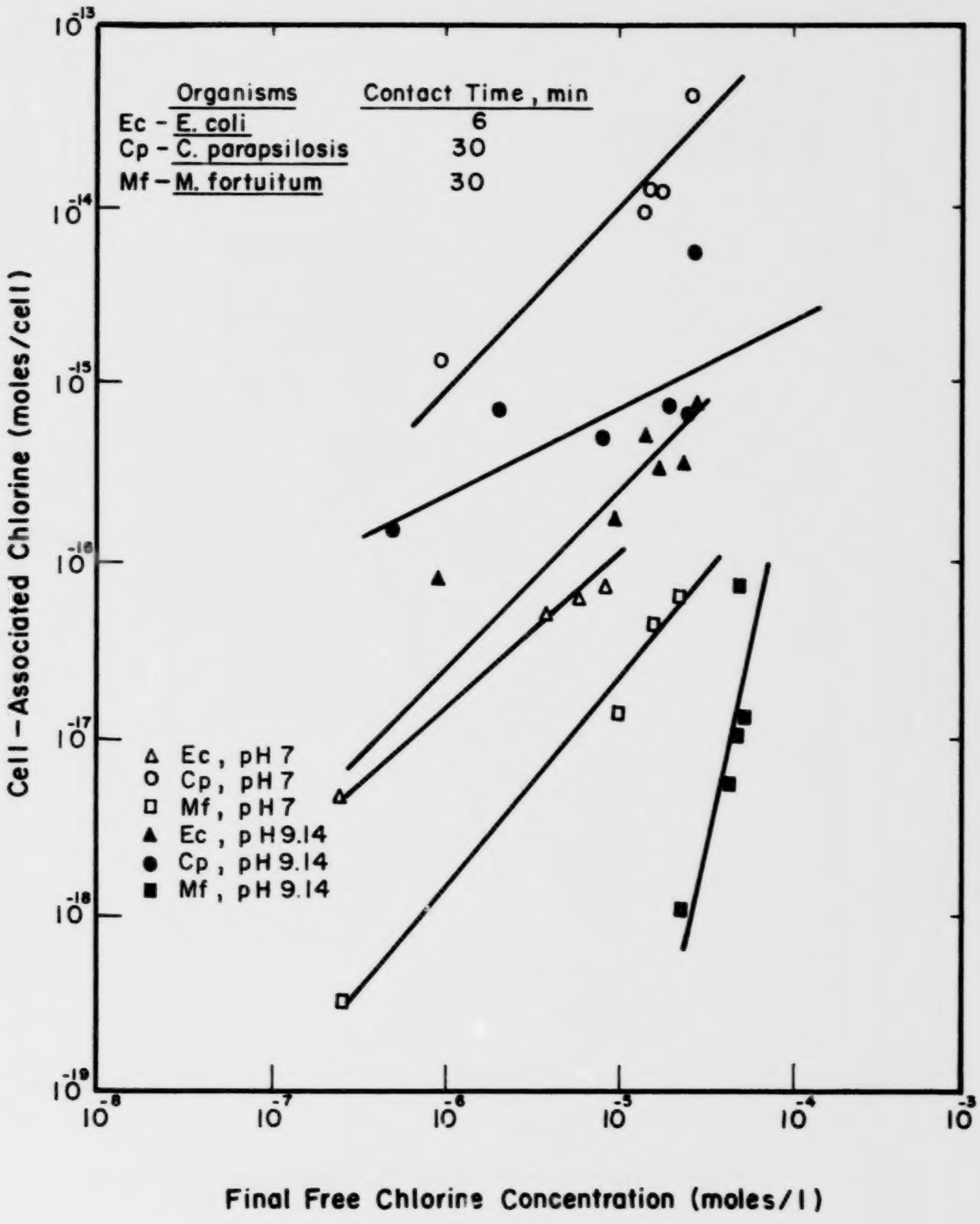


Figure 49. Uptake of Chlorine at Constant Contact Time

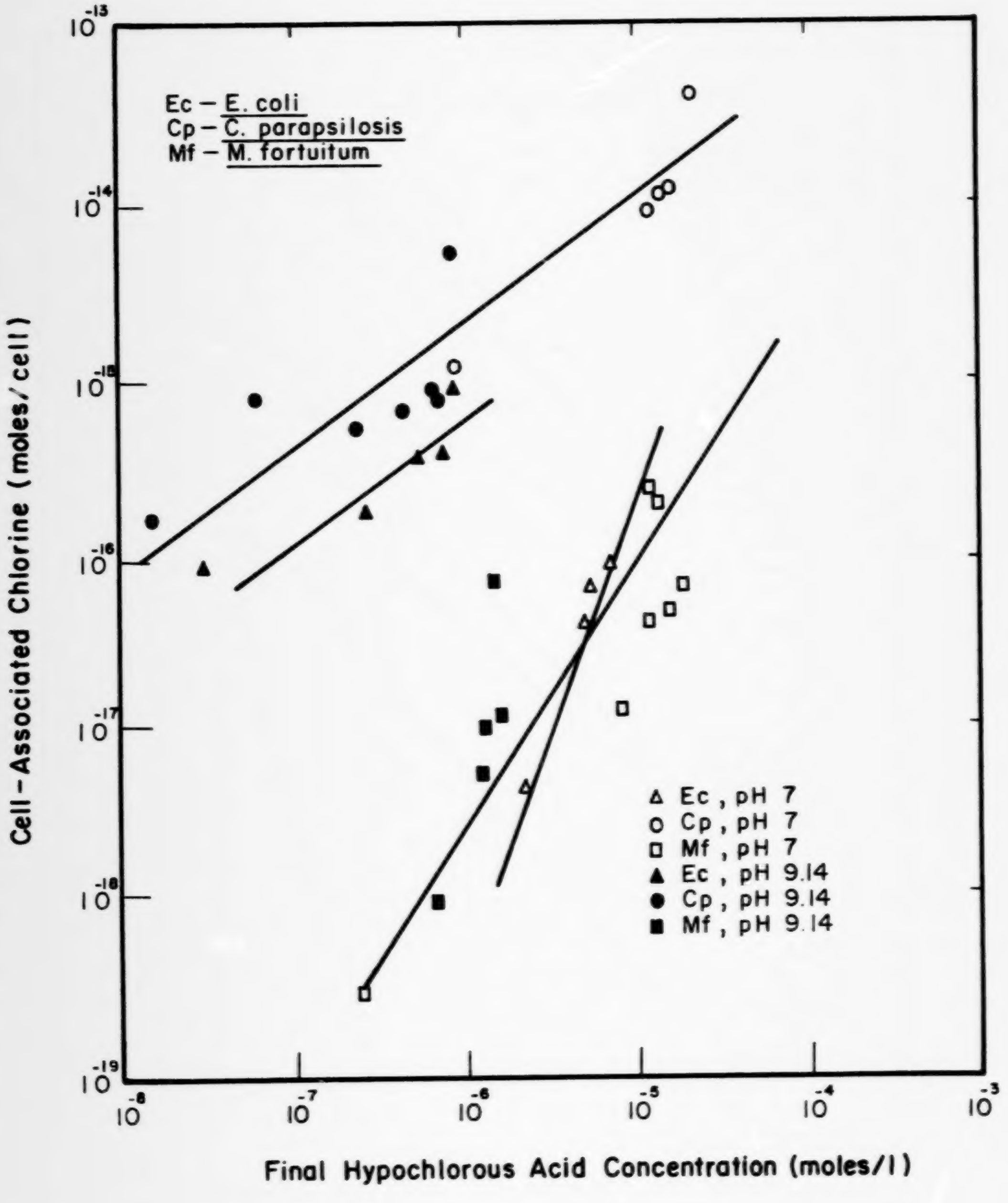


Figure 50. Uptake of Chlorine as HOCl at Constant Contact Time

culture medium and incubated; subsequent growth was monitored using optical density at 660 nm as a criterion.

Results from the growth experiments (Figures 51-53) indicated that microorganisms inactivated by chlorine were rapidly prevented from growing; no initial increase was seen with the treated cells. Only with M. *fortuitum*, where the higher levels of survival were found, was an increase in optical density of chlorine treated cells observed. Even in the face of this result, in no case was any organism able to grow immediately after chlorination-dechlorination. This may be interpreted as showing the lethal event caused by chlorine did not require the participation of growth - death was immediate rather than after a preparatory phase of reproduction. This observation rules out the possibility of a lethal point mutation, such as a base mispair or frameshift, as the principal lethal event. If such mutation were the cause of cell death, it would require at least one generation of replication before the genotype could be expressed. On this basis, it was decided to investigate cell properties most likely to be immediately affected by chlorine.

From the viewpoint of the disinfectant molecule, the first structural feature of the cell contacted, and therefore the first potential lethal site, is the cell wall and cell membrane. Damage to these structures may result in severe damage to cell integrity and/or metabolism, due to a permeability change, or because of a disorganization of respiratory enzymes and transport systems localized near the cell surface. If such damage occurred, the cell may be expected to release ultraviolet-absorbing material, e.g., proteins, nucleic acids or precursors, into solution as well as lower molecular weight organics and inorganic compounds.

Experiments to determine the release of ultraviolet-absorbing material showed that release can occur in certain circumstances. The supernate optical densities for the controls and chlorine treated cells are shown in Table 26. The reported values for percent of total extractables released were calculated by subtracting control optical densities from that of the treated cells and dividing by total extractable material. The latter quantity was measured from the supernatant of an equivalent density of untreated cells boiled for 30 min in pH 7 CDFB. The material released showed a broad absorption band from 250-300 nm; the wavelengths used for calculation were 260 nm, characteristic of nucleic acids, and 280 nm, characteristic of proteins (37). It is of interest that the most resistant test organism, M. *fortuitum*, did not exhibit release of UV material, nor did C. *parapsilosis*, at survival of 2-3 percent, whereas E. *coli* did demonstrate release at 23 percent survival. In the case of conditions where detectable release was observed, confirmation was obtained in at least two replicates of each experiment.

Corroborative evidence that cytoplasmic leakage may occur, following exposure of cells to chlorine, was obtained by measuring supernatant TOC. Results are summarized in Table 27. The primary conclusion from these

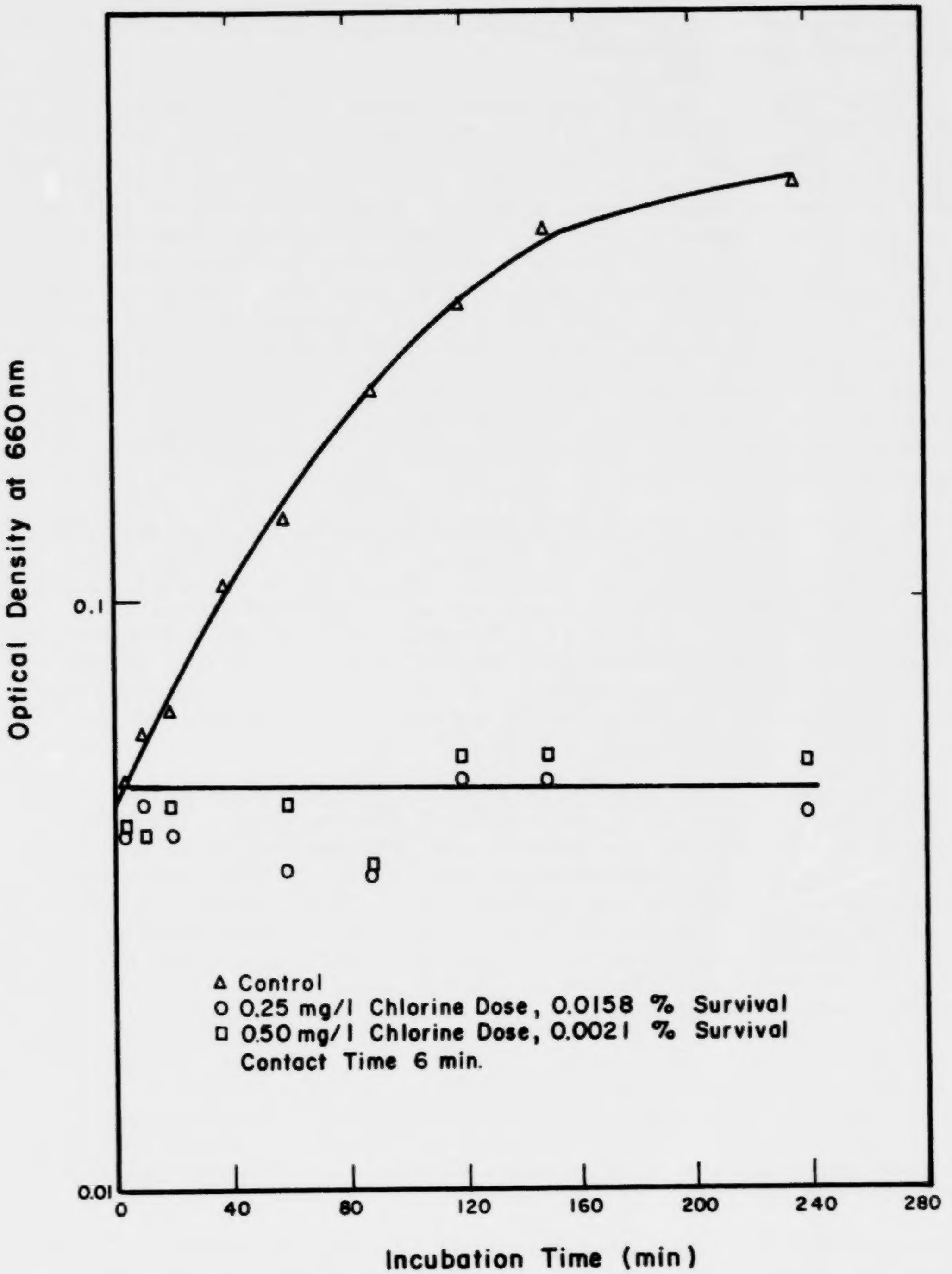


Figure 51. Growth of E. *coli* after Chlorination at pH 7

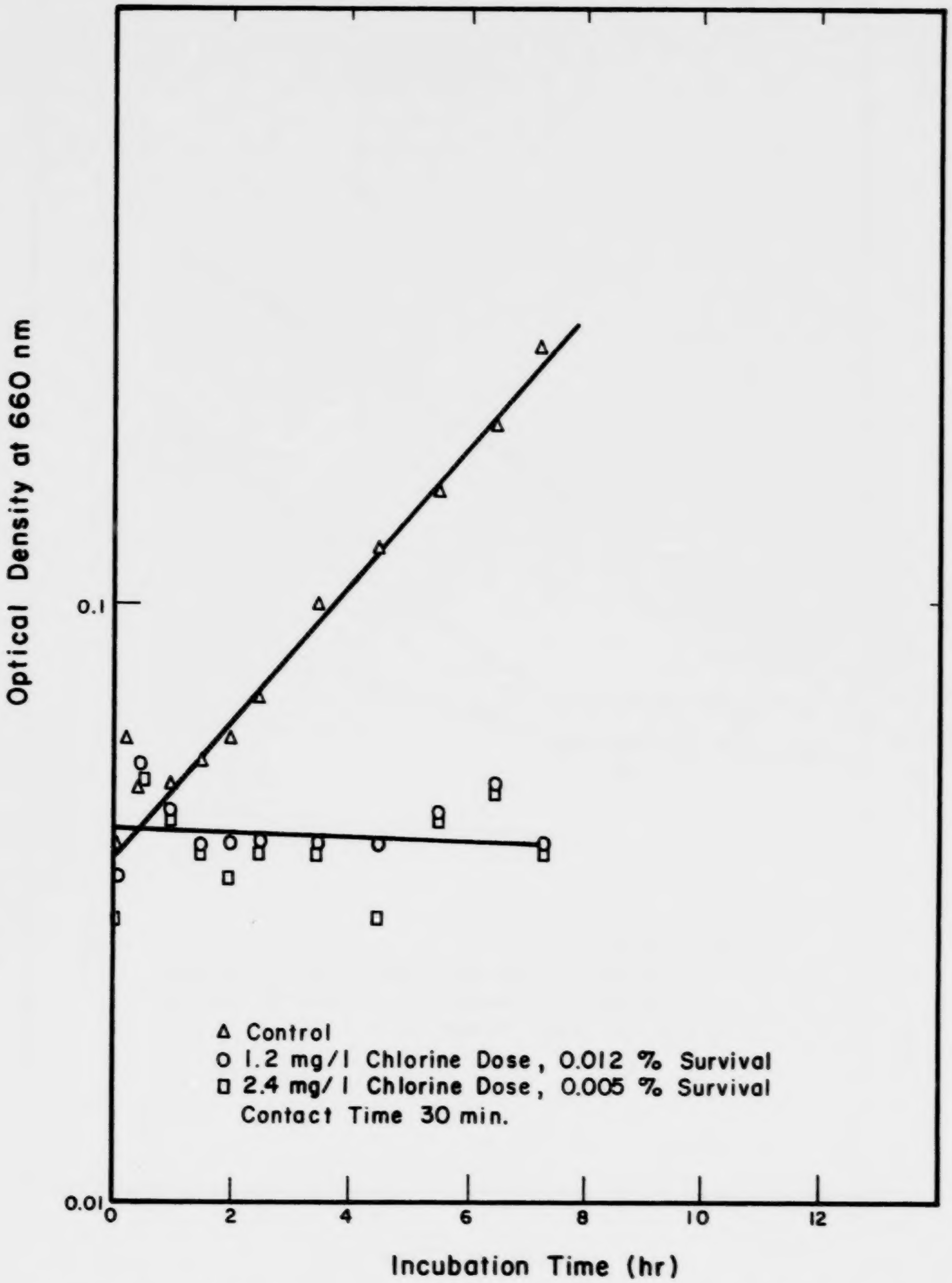


Figure 52. Growth of C. *parapsilosis* after Chlorination at pH 7

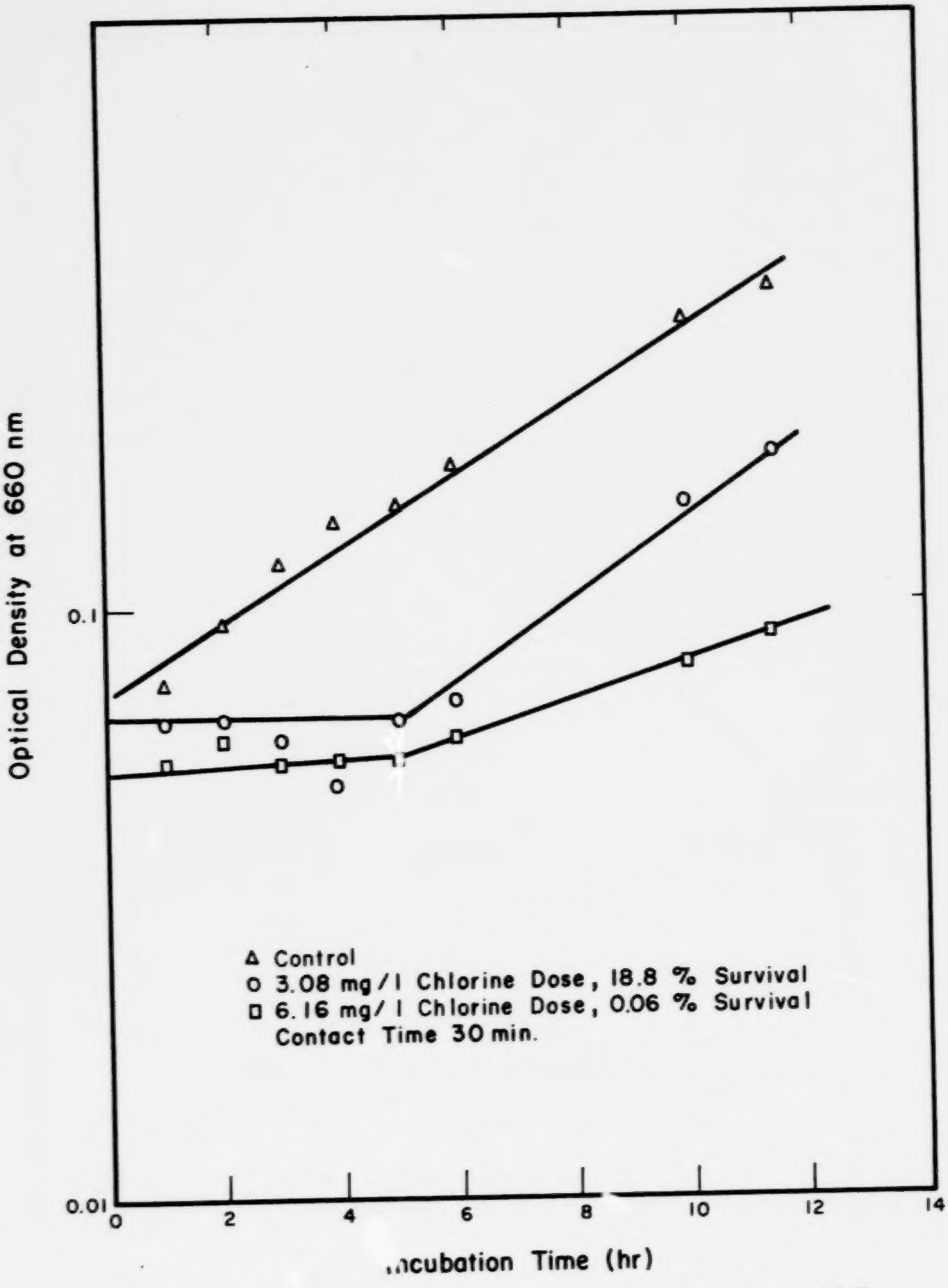


Figure 53. Growth of M. *fortuitum* after Chlorination at pH 7

TABLE 26. RELEASE OF UV ABSORBING MATERIAL AFTER CHLORINATION, pH 7

Organism	Chlorine dose mg/ℓ	Survival at end of experiment %	Optical Density Controls	Optical Density Chlorine treated	Release as % of total UV extractable
		280 nm			
C. *parapsilosis*	1.97	2.3	0.0225	0.011	--
	3.70	*	0.013	0.017	4.7
E. *coli*	0.57	23.0	0.058	0.539	5.0
	3.19	*	0.115	0.128	4.7
M. *fortuitum*	1.93	3.1	0.0515	0.044	--
		260 nm			
C. *parapsilosis*	1.97	2.3	0.024	0.011	--
	3.70	*	0.011	0.025	9.5
E. *coli*	0.57	23.0	0.0566	0.593	3.1
	3.19	*	0.127	0.146	4.9
M. *fortuitum*	1.93	3.1	0.0175	0.006	--

*Survival not measured

TABLE 27. RELEASE OF CELLULAR TOC AFTER CHLORINATION, pH 7

Organism	Chlorine dose mg/ℓ	TOC, mg/ℓ Control	TOC, mg/ℓ Treated	Release as percent of total TOC extractable
C. *parapsilosis*	2.1	0.7	2.9	35
E. *coli*	2.1	5.7	10.3	24
M. *fortuitum*	6.1	4.7	6.3	16

results was that, upon chlorination all three organisms, C. *parapsilosis*, M. *fortuitum*, and E. *coli*, exhibited an increased release of TOC. The concentration of TOC from the control cells may represent a low degree of organic matter in the CDFB, as well as a possible release of material from the metabolizing cells, or from the cell surface. Whether the enhanced TOC released from the chlorine treated cells was from the cell interior or from the cell surface currently is unknown.

The reason the release of UV-absorbing material was not noted with either the acid-fast organism or the yeast at low survival while E. *coli* did release material may relate to the relative impermeability of the cell wall of these organisms. This impermeability may have prevented the cytoplasmic contents from leaking into the medium following chlorination. With the yeast, at high chlorine dose where release was observed, further damage may have occurred to the wall to permit such release.

By comparing the release of percent extractable UV-absorbing material to percent extractable TOC, it was noted that larger percentages of TOC contributing material were released, upon chlorination, than the UV-absorbing material. The measurement of UV-absorbing material probably included large amounts of high molecular weight components, such as proteins and nucleic acids. However, the TOC analyses included these, as well as a large number of low molecular weight substances. The observation that larger percentages of TOC than UV material were released may have indicated that smaller molecular weight organic compounds were preferentially released after chlorination. This observation was in accord with expectations, where the permeability barrier is only partially damaged, and also agreed with the results of Venkobachar *et al.* (28) who observed a sequential release of protein, followed by RNA and DNA, with higher chlorine dosages.

Results of the above studies implicated the cell membrane as the site of action of chlorine. To verify this hypothesis, it was necessary to show that membrane-dependent functions, such as respiration, transport, and synthetic processes dependent on transport were also affected by chlorination.

Studies were performed to ascertain the effect of chlorination on respiration using E. *coli*, C. *parapsilosis*, and M. *fortuitum*. Although some inhibition of oxygen uptake was noted for all three organisms, the results were inconsistent with the hypothesis that enzymatic inhibition at a site within the catabolic pathway is the sole lethal lesion (25). The effects noted could be explained on the basis of damage to cell transport systems during the lethal event itself.

As previously discussed, the TOC and UV release experiments suggested damage to the cell permeability barrier as being of significance in the chlorination process. To ascertain the effects of this disturbance on transport processes associated with the cell membrane, potassium transport was studied. Potassium uptake was selected for investigation since current evidence suggests that nearly all intracellular potassium is present as the free monovalent cation, which is accumulated by an active osmoregulatory mechanism (38). Additionally, potassium transport is readily subject to measurement using radiotracers. It would be expected that the uptake or leakage of potassium by the cell would be highly sensitive to the effects of chlorine at the cell membrane and cell wall, due to the small size of the K^+ ion.

Figures 54 through 56 present the results of the potassium uptake and retention experiments with *E. coli*, *C. parapsilosis*, and *M. fortuitum*, respectively, at pH 7. Upon treatment with chlorine, *E. coli* showed a definite decrease in the amount of potassium associated with the cell. This decrease, about 33 percent following 40 min exposure to chlorine, was relatively greater than that noted for the release of TOC (Table 27) or UV-absorbing material (Table 26). The loss of cellular potassium may be attributed to the action of chlorine promoting leakage through the cell membrane, with a preferential release of lower molecular weight compounds.

With *C. parapsilosis*, the chlorine treated cells showed an initial increase in ^{42}K uptake when compared to the control cells (Figure 55). With prolonged contact, however, the treated cells lost cellular potassium and potassium levels at the end of the experiment were about 40 percent of the control cells. This behavior may have been due to a progressive deterioration in the cell wall leading, at first, to increased permeability of extracellular material; following this, further cell wall or membrane damage may have occurred leading to leakage of the potassium. It should be noted that potassium levels in the experimental cells did not decrease below those of control cells until about 30 min after chlorine addition.

With *M. fortuitum*, the only effect of chlorine upon ^{42}K transport appeared to be a substantial enhancement of uptake (Figure 56). This increase may be due to a partial degradation of the impermeable cell wall of the mycobacteria, leading to greater transport rates. While there appeared to be a decline in the rate of uptake after about 30 min of chlorine contact, no absolute decrease was noted, as in the case of the other organisms.

The differential response of *E. coli* from the other two organisms in the potassium uptake experiments may be explained on the basis of permeability differences. The effect of chlorine on all three organisms may be similar, i.e., change in permeability of the outer layers of the cell. Since *E. coli* has a more permeable cell wall than either *C. parapsilosis* of *M. fortuitum* (39,40), the effect resulted in leakage of material in the former organism, whereas an increase in permeability was seen in the other two organisms.

The release of potassium in *E. coli* and *C. parapsilosis* may be accompanied by the release of anionic compounds, as well, to maintain internal electrical neutrality. One possibility is that anionic organic compounds, such as amino acids, or low molecular weight phosphates, are released simultaneously with the loss of potassium. This phenomenon, similar to that

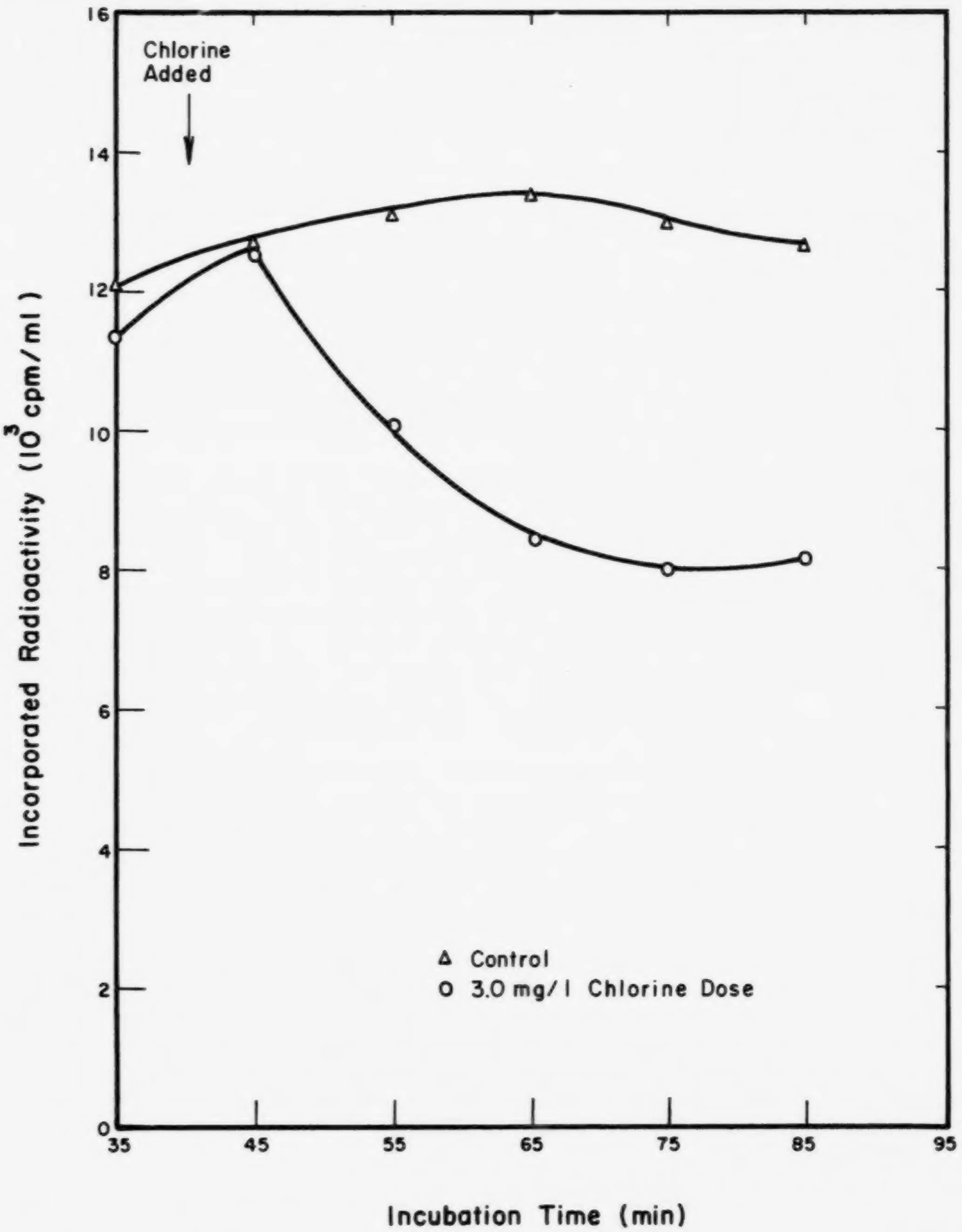


Figure 54. Effect of Chlorine on Cellular Potassium: *E. coli*, pH 7

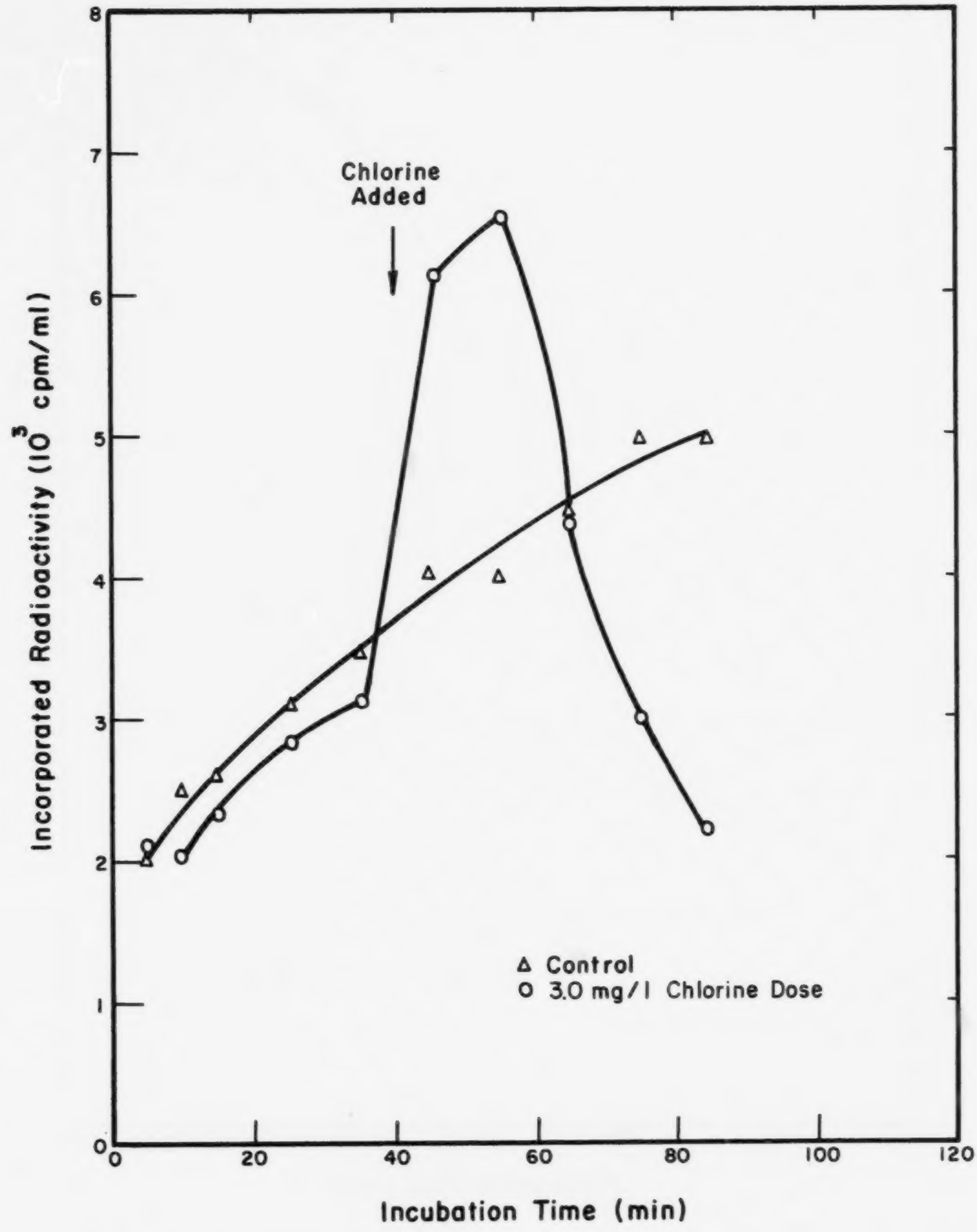


Figure 55. Effect of Chlorine on Cellular Potassium: *C. parapsilosis*, pH 7

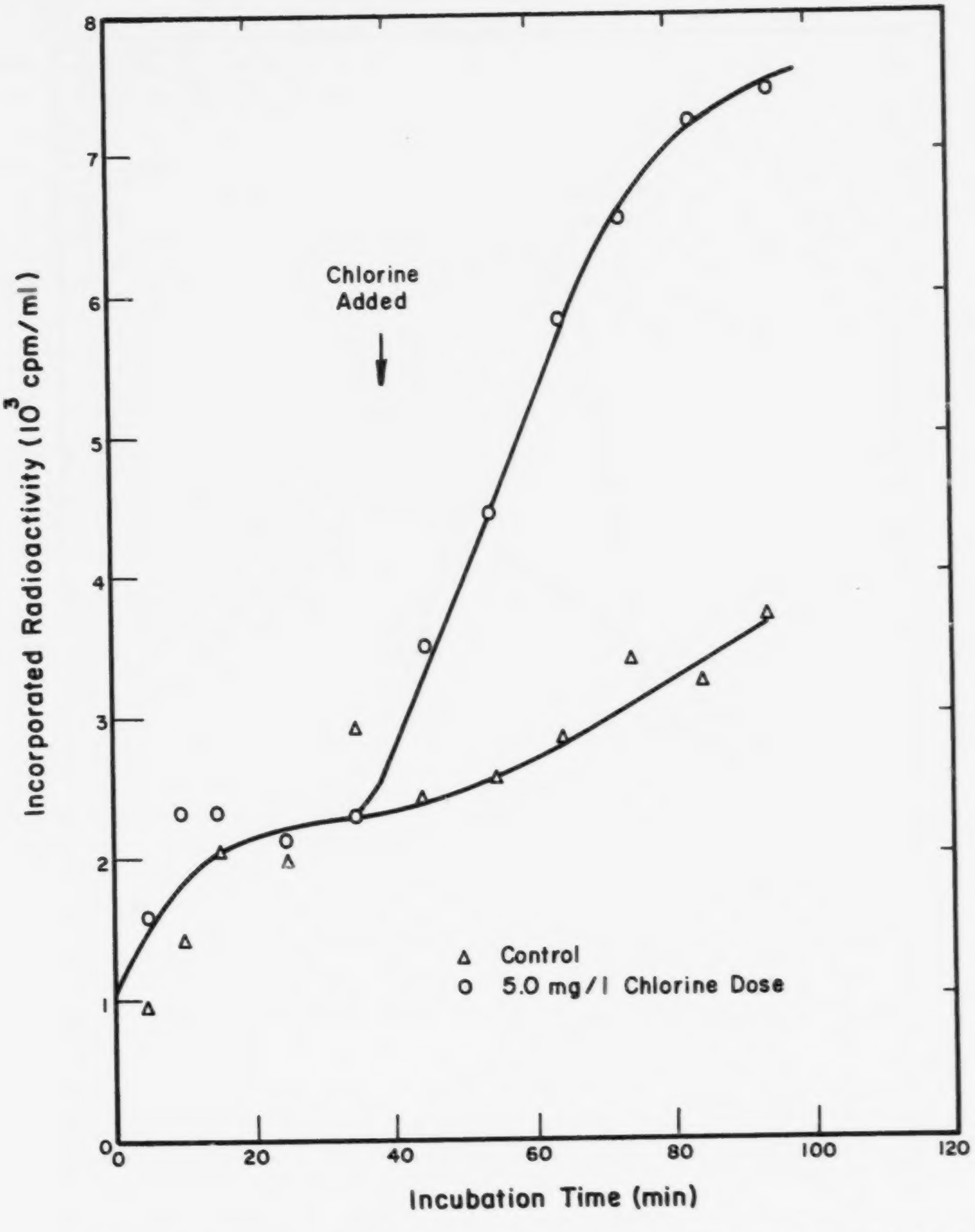


Figure 56. Effect of Chlorine on Cellular Potassium: *M. fortuitum*, pH 7

observed in osmotic downshock of E. *coli* (38), would also account for the observed release of organic materials in this study and for the release of organic compounds (28,41) and phosphate compounds (23) reported in the literature.

Within the overall objectives of this study, the potassium uptake experiments provide primary evidence for the involvement of the cell membrane as a site of action of chlorine. Since the potassium transport system appears to be cellularly controlled (38), and since such control may be exercised at the level of the cell membrane, these data, in addition to those previously described, support cell membrane involvement.

After chlorination, the ability of the three test organisms to synthesize protein appeared to be greatly reduced (Figure 57). This reduction in activity was confirmed, in several cases, by replicate experiments. The observed reduction is identical to that made by Bernarde *et al.* (42) who noted that protein synthesis ceased immediately after treatment of E. *coli* with chlorine dioxide. The apparent blockage of protein synthesis noted in Figure 57 does not, per se, mean that intracellular protein synthesis was stopped. Rather, it may mean that only the ability of the cells to transport the radioactive proline was affected.

As previously noted, the cell permeability barrier is affected as a result of chlorination. The response in protein synthesis may have been due to an impaired transport ability. Alternatively, chlorine may have acted directly on protein synthesis, or the alteration in cellular potassium levels may have interfered with optimum ionic environments for ribosome activity.

The observation that protein synthesis was immediately affected after chlorination was supportive of the findings of the growth experiments (Figures 51-53) in that no growth of the treated cells occurred after chlorination. It apparently was not necessary for any cell growth or synthesis to occur before microbial inactivation.

The effect on protein synthesis provides evidence for an effect of chlorine at a point prior to completion of translation. Using arguments similar to Benarde *et al.* (42), this damage may be at the level of amino acid transport, activation, transcription, or translation. Since an effect on the cell membrane would cause damage to many, if not all, transport processes, this damage, which has already been demonstrated, is sufficient to explain the observed effect.

In the case of C. *parapsilosis*, E. *coli*, and M. *fortuitum*, a lower level of DNA synthesis was observed in chlorine-treated cells than in control cells (Figure 58). An actual decrease in labelled DNA occurred in E. *coli* whereas the other organisms merely showed a reduction in the rate of synthesis.

The cause of this depressed synthesis of DNA may have been the result of an increase in precursor uptake, similar to that stated above for protein

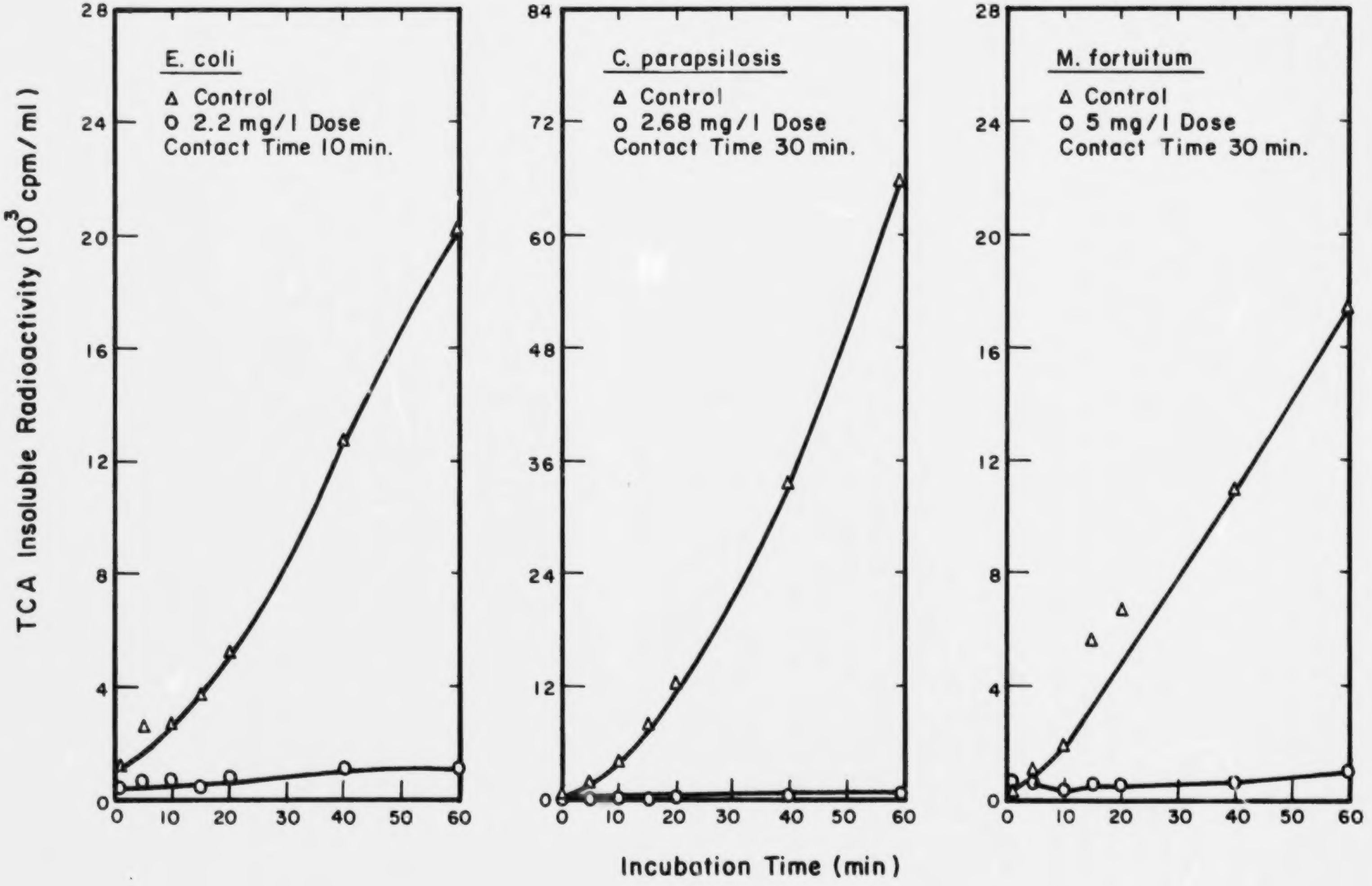


Figure 57. Effect of Chlorination at pH 7 on Protein Synthesis

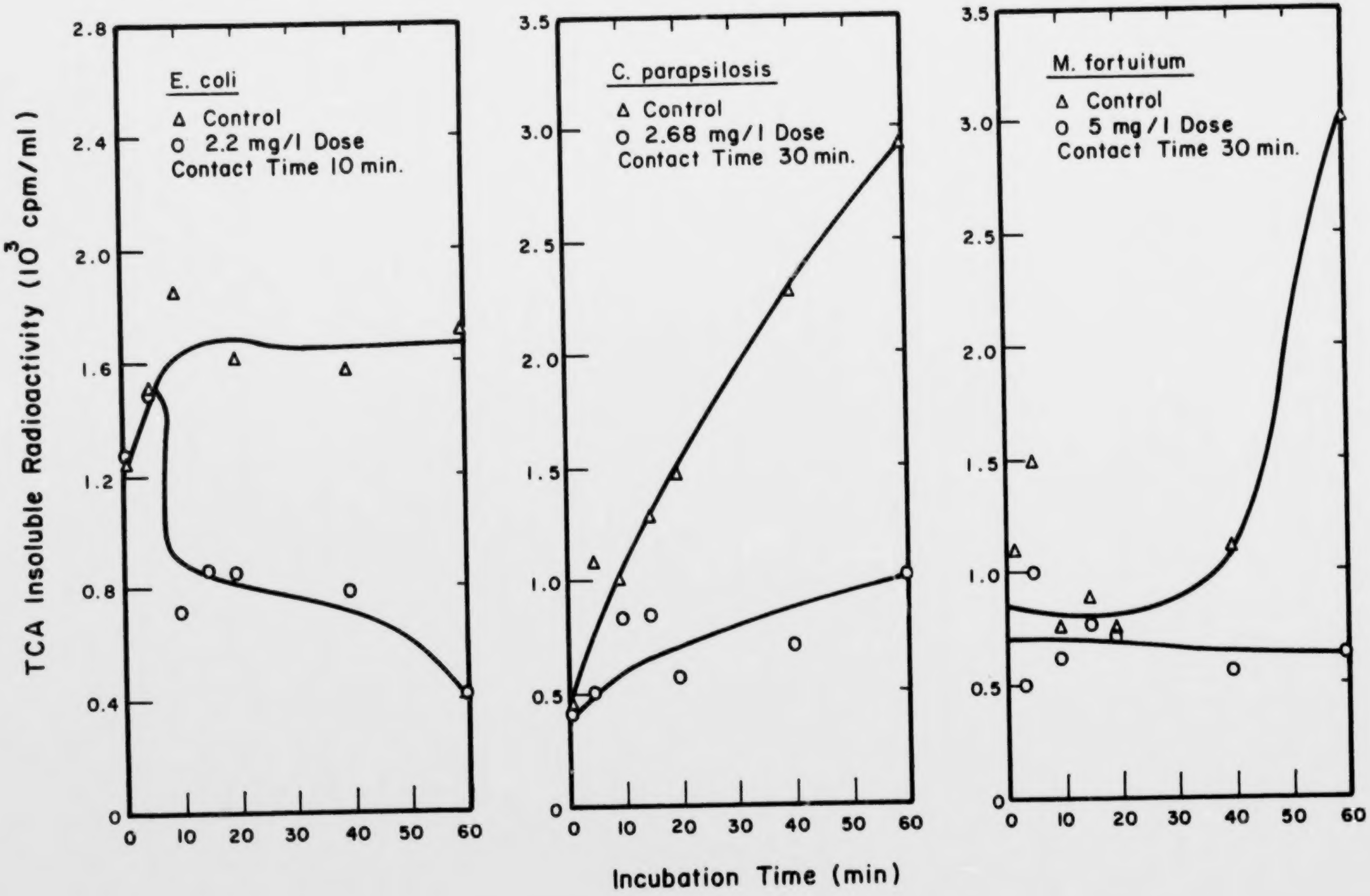


Figure 58. Effect of Chlorination at pH 7 on DNA Synthesis

synthesis. Alternatively, chlorine may have directly interfered with the synthetic process itself, or with the integrity of the DNA template. Observations by Shih and Lederberg (30) point to the formation of single and double strand DNA breaks upon chloramine treatment. These breaks might result in a reduced rate of replication.

The results of the DNA synthesis experiments provide evidence for the involvement of the cell membrane and possibly the DNA, as targets for chlorine damage. As in the case of the effect on protein synthesis, membrane damage leading to loss of transport activity would produce the observed effect.

Lethal Lesions

As noted earlier, discussion of the mode of action of chlorine can be reduced to two questions. First, is the toxic action dependent on penetration of the disinfectant molecule into the cell, and, if so, is this the basis for the increased efficiency of hypochlorous acid, vis-à-vis hypochlorite ion? Second, what are the lethal lesions caused by chlorine?

On the basis of the results, plus a literature review, chlorine appears to be capable of producing lethal events at or near the cell membrane as well as in respect to DNA. The primary evidence for the involvement of the cell membrane as a site for the lethal action of chlorine was gathered from the results of the TOC release, UV-absorbing material release, potassium uptake, protein and DNA synthesis, and respiration studies. The results of the TOC release (Table 27) and the UV-absorbing material release (Table 26) experiments indicated that, after exposure to chlorine, all three organisms exhibited some level of release of either or both of these materials; the only exceptions were in the UV-absorbing material release studies of *C. parapsilosis* with low dosages of chlorine, and *M. fortuitum*. Furthermore, in the potassium uptake studies, both *E. coli* and *C. parapsilosis* exhibited lower levels of intracellular potassium after chlorine treatment (Figures 54 and 55). The increase in potassium levels observed with *C. parapsilosis* immediately after chlorine addition and with *M. fortuitum* (Figures 55 and 56) may have been due to an initial degradation of the thicker and less permeable cell wall in both of these organisms, permitting a greater permeability of ^{42}K; after this initial phase with *C. parapsilosis*, the chlorine may have degraded the cell membrane further, resulting in a loss of material and greater levels of cell inactivation. The effects noted on protein and DNA synthesis (Figures 57 and 58) were consistent with a disruption of the cell membrane as a permeability barrier.

Supporting evidence for the involvement of the cell membrane as a lethal target was seen in the growth experiments (Figures 51-53). The immediate cessation of growth after chlorine treatment was consistent with a direct physical damage, such as a membrane effect, rather than a lethal point mutation, or similar lesions requiring growth prior to expression.

In summary, therefore, the lethal lesions caused by chlorine appear to be a disruption of the cell membrane and cell permeability, and possibly a physical damage to the DNA of the cell. Based on the research results presented, the permeability hypothesis of differential action of HOCl and OCl^- of Chang (18) has been verified in vegetative microorganisms.

Permeability Hypothesis

The primary evidence for the acceptance of the permeability hypothesis are the results obtained from the isotherm studies (Figures 49 and 50). From these data, with both C. *parapsilosis* and M. *fortuitum*, it was noted that more chlorine was found in association with the cell after exposure at low pH than after exposure at high pH. Furthermore, the amount of chlorine found in association with these cells appeared to be based predominantly upon the theoretical concentration of undissociated hypochlorous acid in solution, as would be predicted. The results for E. *coli* confirmed the observations of Friberg (22,23) that more chlorine was found in association with the cells at high pH than at low pH and, at first glance, would appear to negate the permeability theory. However, since it has already been demonstrated that the action of chlorine may have resulted in the loss of considerable cellular material, especially with the more sensitive E. *coli*, it is entirely possible that the increased efficiency of chlorine at low pH promoted extensive loss of cell material, including associated chlorine, leading to an underestimate of the latter quantity at low pH. Thus, the current work appears to extend the permeability theory of chlorine action to vegetative microorganisms in addition to those previously described.

To answer the questions posed at the outset of this phase of study, the mode of action of free available chlorine in inactivating microorgansims appears to be penetration into the cell, at least through the outer cell wall. Following this, the chlorine may attack the cell membrane and cause cell permeability disruption, including loss of cytoplasmic constituents, as well as secondary metabolic disturbances. An additional primary lethal lesion may be the physical degradation of DNA.

Resistance of Yeast and Acid-Fast Organisms

Considering the possible mechanism whereby chlorine inactivates microorganisms, the increased resistance of the acid-fast and yeast organisms may be contrasted to that of E. *coli*.

Comparing cell size, C. *parapsilosis* has about 20-100 times the volume and surface area per cell as E. *coli*. At pH 9.14 C. *parapsilosis* takes up only about 2-10 times the amount of chlorine as E. *coli*; after correction for increased leakage, a similar ratio may be calculated for low pH values as well. It thus appears that one possible reason for the increased resistance of the yeast C. *parapsilosis* may be a decrease in its ability to take up chlorine from solutions of equivalent concentration. This possibility may be related to a difference in the nature and composition of the cell wall of the two different organisms. From a priori considerations, Cooke (40) predicted that yeasts would be more resistant to chlorine than E. *coli* on the basis of decreased permeability. It would appear, therefore, that the ability of yeast to take up less chlorine may be due to a heightened permeability barrier.

The decreased ability of bound chlorine to cause lethal events may also be due to the distinctive features of the cell wall of yeasts, i.e., thickness of the cell wall. A key event in cell inactivation would appear to be the loss of material from the cell. The thickness of the cell wall in yeasts, far in excess of that associated with E. *coli*, may be much more resistant to lesions causing cytoplasmic leakage (43).

E. *coli* and M. *fortuitum* are of similar size and would, therefore, be be expected to take up a similar quantity of chlorine. On the other hand, it is clearly apparent from the results of this study that M. *fortuitum* takes up considerably less chlorine than does E. *coli*. This, in all probability, reflects the very high lipid level in the cell walls of the acid-fast bacteria, making them very impermeable to polar molecules, e.g., hypochlorous acid (39,44).

The impermeable cell wall of the mycobacteria may significantly reduce the loss of cytoplasmic material. In the UV release experiments, no release of UV-absorbing material was detected even though 97 percent inactivation with chlorine occurred (Table 26). One interpretation may be that the cell wall plays an important role in resistance by retaining small amounts of material lost through the cytoplasmic membrane.

As a result of the above studies, it may be concluded that the increased resistance of yeasts, such as C. *parapsilosis*, to free available chlorine is primarily a result of the thickness and rigidity of the cell wall. The increased resistance to free available chlorine exhibited by acid-fast organisms, such as M. *fortuitum*, probably results principally from the impermeability of the cell wall. Both of these organisms, therefore, may be expected to be more resistant than E. *coli* to chlorine in a wide variety of circumstances.

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APPENDIX A

THE RELATIVE RESISTANCE OF ACID-FAST AND OTHER ORGANISMS TO CHLORINATION: A REVIEW

INTRODUCTION

In view of the apparently lower resistance of coliforms to disinfection than certain pathogens, notably enteric viruses, many workers have attempted to find a new and more appropriate indicator of fecal pollution and/or disinfection efficiency (1). One group of organisms proposed has been the acid-fast bacteria.

In order to be useful as an indicator of disinfection efficiency, the proposed organism must be at least as resistant as the most resistant pathogen. Studies in this laboratory have demonstrated that the acid-fast organisms are considerably more resistant to chlorination than either coliforms, poliovirus type 1 or *Salmonella typhimurium*. Field work in progress also appears to show that removal and/or die-off of acid-fast bacteria in wastewater treatment processes and downstream from an outfall is less than conventional indicator organisms, i.e., coliform organisms.

In many parts of the world, *Mycobacterium tuberculosis* is an important pathogen. Sanitoria wastes and domestic wastewaters can contain significant densities of the tubercle bacilli (2-4). Natural waters with and without wastewater effluents also contain significant acid-fast bacteria (5). It appears that these organisms may be excreted by healthy individuals (6,7). If acid-fast organisms, other than M. *tuberculosis*, as a group are more resistant to disinfection than M. *tuberculosis*, then their usefulness as indicators of disinfection efficiency would be enhanced. It is the purpose of this review to determine this relative resistance.

RESISTANCE OF ACID-FAST ORGANISMS TO CHLORINATION

The resistance of acid-fast organisms to chlorination has been studied by Engelbrecht and co-workers (1,6). Several acid-fast species were isolated from samples of chlorinated secondary wastewater effluent, including M. *fortuitum* and M. *phlei* (6). Early work with these isolates showed that they were, especially M. *fortuitum*, more resistant to free-available chlorination than a variety of human enteric viruses (Figure 1).

Chloramines are much weaker disinfecting agents than the forms of chlorine measured as free residual. Studies currently being completed with chloramines (8) showed that M. *fortuitum* is also significantly more resistant to the effects of this form of chlorine than coliform organisms.

RESISTANCE OF *Mycobacterium tuberculosis* TO CHLORINATION

In reviewing the early work on the subject, Greenberg (9) discussed the survival of tubercle bacilli in wastewater treatment processes. Starting from the date of his review, a comprehensive search of the literature was performed for information on the sensitivity of M. *tuberculosis* to chlorination.

Rhines (10), using Avian tubercle bacilli, found that a dose of 91 mg/ℓ of chlorine effected 99.96 percent inactivation within 30 min. In this and other examples, it will be assumed that the minimum quantifiable level of M. *tuberculosis* is 1 organism/mℓ in order to calculate inactivation levels.

Jensen and Jensen (11), working with secondary effluent obtained from a tuberculosis sanitorium, observed that contact with a dose of 20 mg/ℓ of chlorine for 40 min resulted in over 99.99 percent inactivation of the tubercle bacilli; a dose of 9 mg/ℓ for 1 hr resulted in over 99.999 percent inactivation.

In later work, Jensen (12,13) measured the chlorine dose and residual necessary to inactivate 99.999 percent of the tubercle bacilli in raw wastewater and secondary effluent. Chlorine residual measurements were performed by the "OTD" method.

Heukelekian and Albanese (14) measured the orthotolidine residual necessary to inactivate 99.9 percent of a culture of tubercle bacilli in sterilized raw wastewater. Pramer *et al.* (15) measured the "free residual" and orthotolidine chlorine residual needed to inactivate tubercle bacilli in secondary effluent.

More recently, Bhaskaran *et al.* (3) studied the chlorination of treated sanitoria effluents. Residuals were measured by an unspecified technique.

Dozanska and Manowska (16) found identical sensitivity of the $H_{37}Rv$ and BCG strains of M. *tuberculosis* to chlorine residuals. The effects of free and combined chlorine were anlayzed separately. Free chlorine was measured by the OT and OTA methods while total residuals were controlled using the DPD procedure. Chlorination results were reported for "100 percent" inactivation.

In a later study, using the BCG strain, Dozanska and Manowska (17) studied the chlorination of untreated raw wastewater monitored by the OTA method until "destruction of tubercle bacilli approached 100 percent."

A summary of the data reported by the above researchers appears in Table 1.

Figure 2 summarizes the data from Table 1 for raw wastewater, Imhoff or septic tank effluent, compared with the inactivation of M. *fortuitum* in

TABLE 1

REPORTED CONDITIONS FOR 100 PERCENT INACTIVATION OF M. *tuberculosis* BY CHLORINE

Suspending Menstrum	Raw Wastewater, Imhoff or Septic Tank Effluent	Secondary Effluent	Tap Water or Buffer
Dose	Musehold (18) 63 ppm 2 hr	Jensen & Jensen (11) 20 ppm 40 min 9 ppm 1 hr	Kopeloff & Davidoff (20) 30-50 ppm 5 min
	Sollazzo (19) 20 ppm 2 hr 33.6 ppm 25 min	Jensen (12,13) 10 ppm 1 hr	Green (21) 125 ppm 10 min 65 ppm 30 min 35 ppm 4 hr
	Jensen (12,13) 25 ppm 1 hr		
	Rhines (10) 91 ppm 30 min		
Residual	Jensen (12,13) 3.5 ppm 1 hr	Jensen (12,13) 0.4-0.95 ppm 1 hr	Dozanska & Manowska (16) (see Figure 4)
	Dozanska & Manowska (16) (see Figure 5)	Heukelekian & Albanese (14) 2 ppm 1 hr 1 ppm 2 hr	
	Dozanska & Manowska (17) 5 ppm 1 hr	Bhaskaran *et al.* (3) 0.9 ppm 1 hr	
		Pramer *et al.* (15) 0.9 ppm (free) 30 min 2-5 ppm (OT) 30 min	

phosphate buffer by both free-available chlorine (1) and chloramines (8). Figure 3 depicts the resistance of the tubercle bacilli in secondary effluent and, again, compared to inactivation of M. *fortuitum* in phosphate buffer.

The most comprehensive study reported has been that of Dozanska and Manowska (16). They investigated the inactivation of tubercle bacilli to chlorine in tap water and wastewater, using four pH values and two temperatures. The results of their work, compared with results from this laboratory for both free-available chlorine and chloramines, are shown in Figures 4 and 5, respectively.

DISCUSSION OF COMPARATIVE RESISTANCE TO CHLORINE

As Figures 2 and 3 indicate, the data for the inactivation of M. *tuberculosis* by chlorine in raw wastewater and secondary effluent reported by others are bracketed by our observations with M. *fortuitum*. This would seem to indicate that there is no evidence to support the proposition that the tubercle bacilli are more resistant to chlorination than the more resistant acid-fast organisms such as M. *fortuitum*.

As would be expected, the data given for chlorine dosage in Table 1 fall significantly above our data using residual measurements. In most of the studies with raw wastewater, Imhoff and septic tank effluent and secondary effluent the dosages are in such a range that little or no free residual would be expected. The only inconsistent data are those of Green (21) which seem to be at chlorine dosages in excess of what would be required to reach the breakpoint in tap water or buffer. However, inasmuch as the exact details of his experimental systems were not given, further comment is unwarranted.

Dozanska and Manowska (16) appear to have carried out the most thorough studies. Examination of Figures 4 and 5, which compares their data for 100 percent inactivation of M. *tuberculosis* with data of Severin (8) and Engelbrecht *et al.* (1) for 99.9 percent inactivation of M. *fortuitum* and M. *phlei*, seems to indicate that while M. *fortuitum* and M. *tuberculosis* show similar slopes of time vs. concentration, M. *tuberculosis* is more sensitive to chlorine and chloramine than M. *fortuitum*.

As can be seen in Figure 4, M. *phlei*, while obeying a similar time-concentration slope as the other acid-fast bacteria, is less resistant than M. *tuberculosis*. For a given contact time, it appears that approximately twice the free chlorine residual required for M. *phlei* is needed for inactivation of M. *tuberculosis* for a given contact period.

In the case of combined chlorine, there is still greater discrepancy. With a contact time in excess of 30 min, more than 2.5 times the chlorine residual required for inactivation of M. *phlei* is necessary to inactivate M. *tuberculosis*. On the other hand, about 125 percent of the combined chlorine residual required to inactivate M. *tuberculosis* is necessary to inactivate M. *fortuitum* for a given contact time.

RELATIVE RESISTANCE TO OTHER TOXIC AGENTS

In an extensive review of mycobacteria inactivation, Croshaw (22) noted that a number of researchers believe that "atypical" mycobacteria have greater heat tolerance than M. *tuberculosis*. Croshaw, in analyzing the information, restated a hypothesis that resistance to chemical antagonists was approximately proportional to cellular lipid content. This would imply a higher resistance by the high lipid tubercle bacilli than by the more saprophytic species of mycobacteria.

At least one study appears to have shown that natural die-off of the tubercle bacilli is greater than other acid-fast bacteria. Savov (23) found that, under similar conditions in Lowenstein-Jensen medium, M. *tuberculosis* survived up to 2.5 yr whereas M. *avium* and M. *fortuitum* each survived up to 13 yr.

Further support of this conclusion arises from the observation of Beerwerth (7) that Runyon's Group I, which included the pathogenic mycobacteria, were not present when numerous other mycobacteria were isolated from surface waters and "pasture watering tanks."

CONCLUSIONS

The data gathered in this analysis indicate that at least some acid-fast organisms present in domestic wastewater may be more resistant to chlorination than M. *tuberculosis*. If these organisms, such as M. *fortuitum*, were present and detectable at significantly high levels in wastewaters and raw water supplies, then the acid-fast group of organisms could be useful as indicators of the disinfection of tubercle bacilli.

If, however, organisms such as M. *fortuitum* were absent, and only less resistant acid-fast organisms, such as M. *phlei*, were present, the acid-fast organisms may not be particularly useful as indicators of the disinfection of tubercle bacilli.

The apparent resistance of M. *fortuitum* to chlorination, because of its lower lipid content, is in apparent contradiction to the hypothesis presented by Croshaw (22). Thus, there should be no attempt to extrapolate the findings of this review to relative resistance towards other disinfecting agents, e.g., ozone, iodine, etc.

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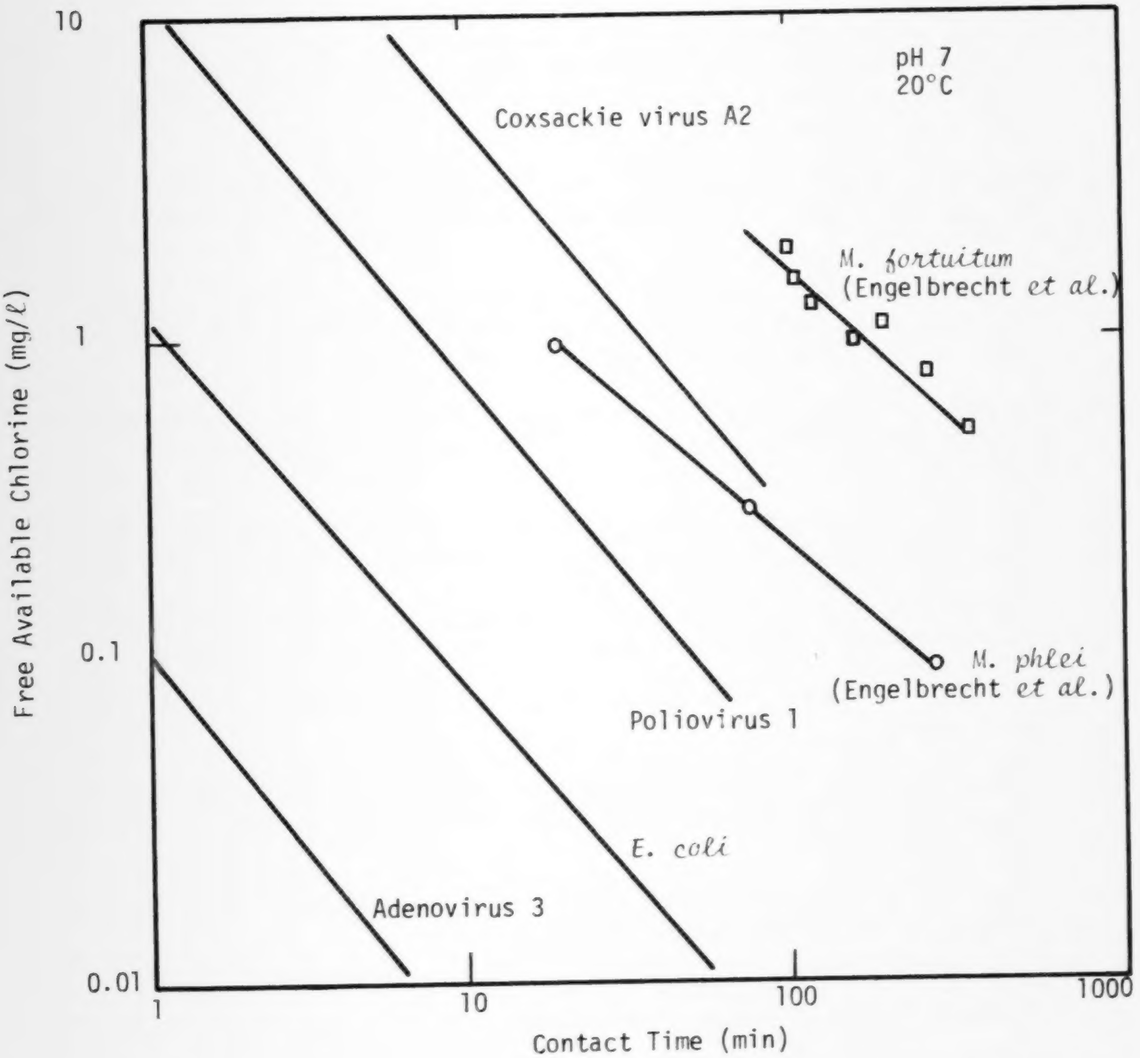

Figure 1. Free Chlorine Residuals and Contact Times Necessary for 99.9 Percent Kill of *M. fortuitum* and *M. phlei*, Compared with Data for Other Organisms Assembled by Berg (24)

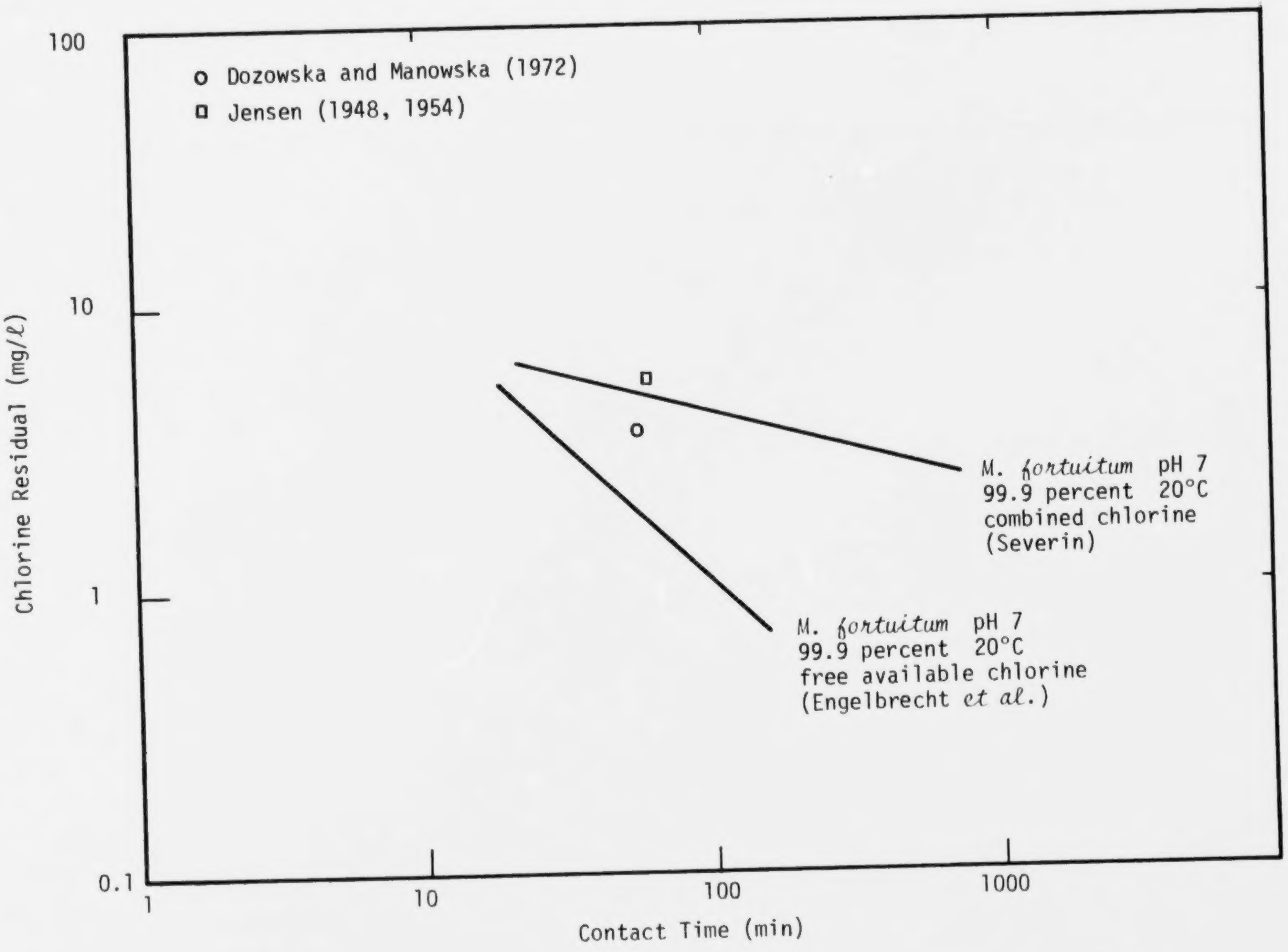

Figure 2. Comparison of Data for Inactivation of M. *tuberculosis* in Raw Wastewater

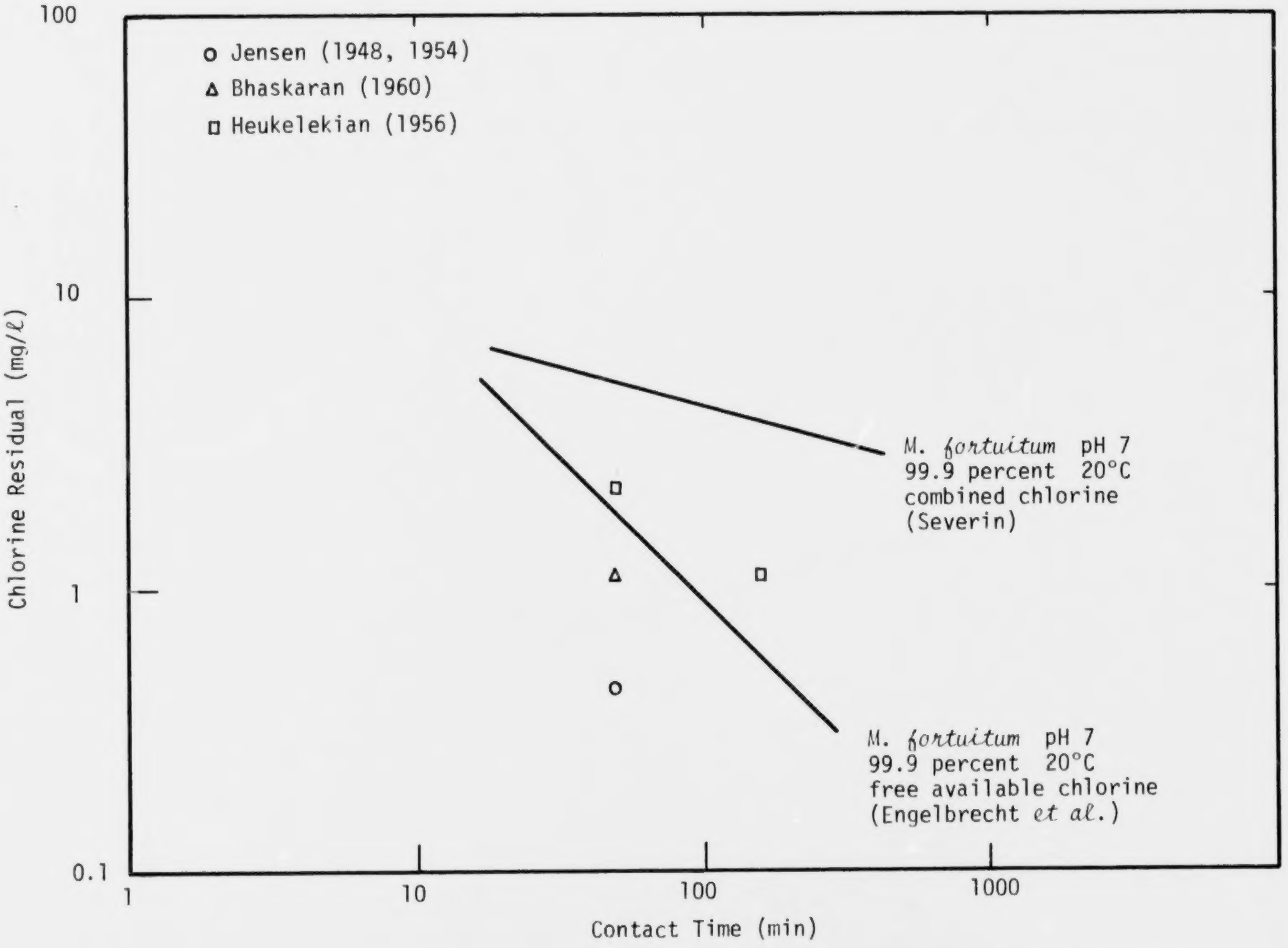

Figure 3. Comparison of Data for Inactivation of M. *tuberculosis* in Secondary Effluent

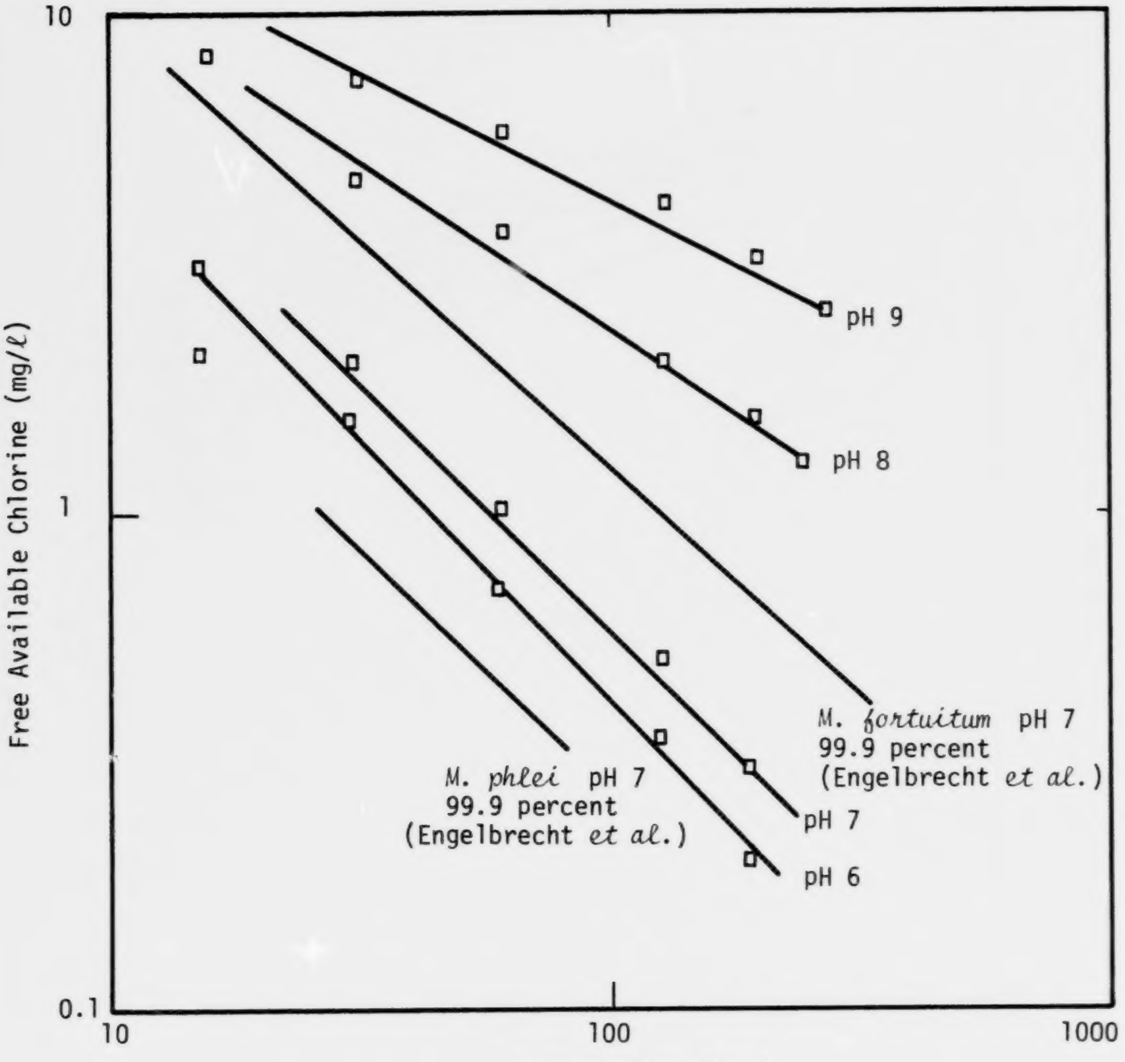

Figure 4. Comparison of Data of Dozanska and Manowska (1970) for 100 Percent Inactivation of M. *tuberculosis* by Free Available Chlorine at 20°C

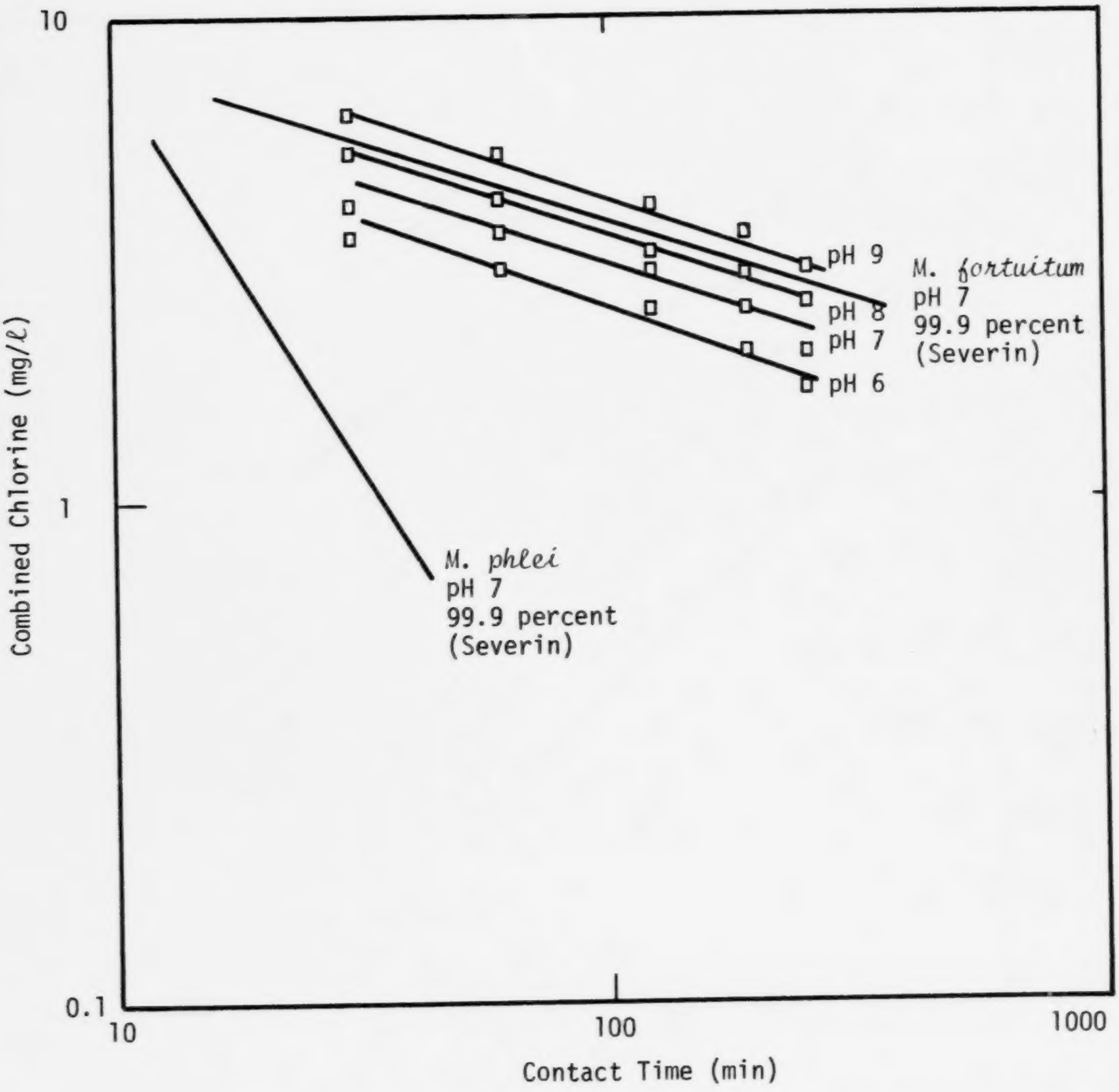

Figure 5. Comparison of Data of Dozanska and Manowska (1970) for 100 Percent Inactivation of M. *tuberculosis* by Chloramines at 20°C